Contribution of ABC Transporters to Antiepileptic Drug Resistance in Temporal Lobe Epilepsy

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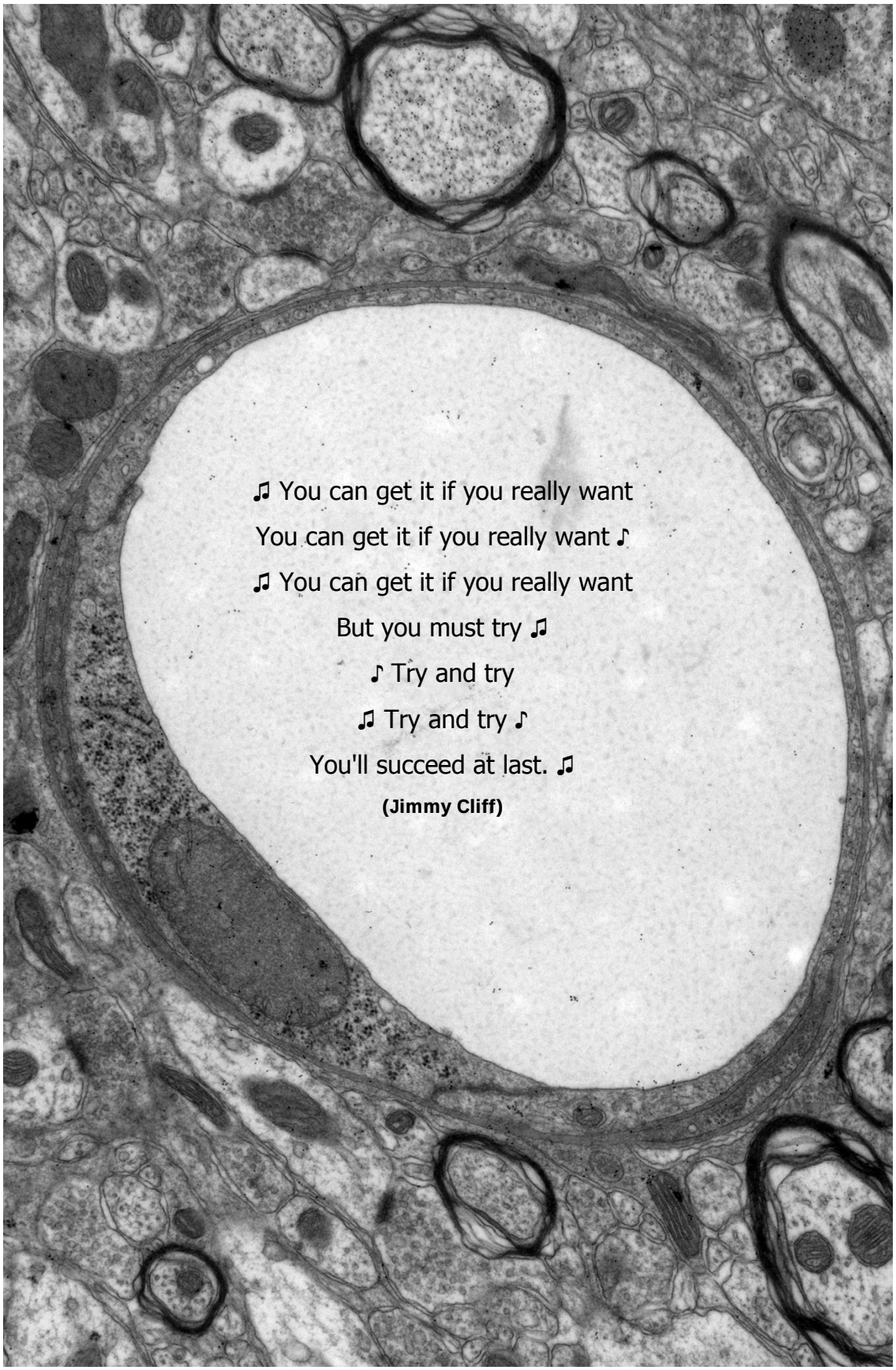

Electron micrograph of cross section of a brain capillary, the site of the blood-brain barrier.

Picture by Caro Petitjean (the master of EM pics!)

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1 Zusammenfassung

Temporallappenepilepsie (TLE) verbunden mit hippokampaler Sklerose ist eine weit verbreitete Erkrankung des Zentralnervensystems. Häufig ist bei dieser Form der Epilepsie die Behandlung der Anfälle durch eine so genannte Antiepileptika-Resistenz erschwert, wenn nicht gar gänzlich verunmöglicht. Die Ursachen dieser Pharmakoresistenz sind unklar. Eine Hypothese, die Transporter-Hypothese, besagt dass eine verstärkte Beseitigung von Antiepileptika aus dem epileptischen Fokus zu einer unzureichenden Medikamentenkonzentration führt. Der Abtransport der Medikamente an der Blut-Hirn Schranke (BHS) läuft vermutlich über sogenannte Multidrug-Transporter, vor allem der ABC-Transporter Familie. Es ist daher wichtig zu untersuchen, ob ein Antiepileptika ein Substrat für solche ABC-Transporter ist, um vorhersagen zu können, wie die Medikamente unter physiologischen wie auch pathologischen Bedingungen im Hirn verteilt sind. Dies ist die Grundlage, um zukünftig Medikamente mit verbesserter Penetration des Gehirns und stärkerer Wirksamkeit zu entwickeln. In dieser Arbeit wurde untersucht, welche Rolle Multidrug-Transporter der ABCB, sowie der ABCC Familie, in der Pharmakoresistenz gegen Antiepileptika spielen. Wir benutzten einen *in vitro* sowie einen *in vivo* Ansatz.

Einerseits haben wir ein neuartiges *in vitro* BHS Modell entwickelt. Dieses besteht aus polarisierten Epithelzellen (MDCK Zellen) welche mit einer, mittels Doxycyclin induzierbaren, Expressionskassette für die humanen ABC-Transporter ABCB1 und ABCC1 stabil transfiziert wurden. Die Zellen bilden dichte Monolayers. Daher wird ein sogenanntes Zwei-Kompartiment-System mit zwei getrennten Hälften gebildet, wenn die Zellen auf einem Filtereinsatz wachsen. Der Zellrasen bildet dabei eine selektive Barriere. Nachdem monoklonale, stabil transfizierte Zelllinien erzeugt worden waren, wurden in diesem System Transportstudien durchgeführt. Zuerst verifizierten wir die induzierbare Expression und korrekte Lokalisation der rekombinanten Transporter mittels Western Blot und Antikörperfärbung der Zellen, dann überprüften wir die Funktionsfähigkeit, indem wir den Transport eines bekannten Substrats testeten. Für die Transportstudien wurde, eine bestimmte Zeit nach der Zugabe eines Stoffes in eines der Kompartimente, die Stoffmenge in den verschiedenen Kompartimenten mittels LC-MS/MS ermittelt. Durch den Vergleich des Transports in Zellen, welche den Transporter überexprimieren, mit dem in nicht-induzierten Zellen, wurde ermittelt, welchen Anteil die überexprimierten Transporter an den Gesamttransport haben. Für

die beiden getesteten Antiepileptika Carbamazepin (CBZ) und Lamotrigin konnte kein Transport, weder durch ABCB1 noch durch ABCC1, gezeigt werden. Hieraus wurde geschlossen, dass beide entweder keine oder nur sehr schwache Substrate der getesteten humanen ABC-Transporter sind. Erstaunlicherweise wurde jedoch beobachtet, dass alle getesteten Substanzen, unabhängig von der Expression der induzierbaren ABC-Transporter, aktiv (da stärker bei 37°C als bei 4°C) durch die MDCK Zellen transportiert wurden und zwar in basolateral-nach-apikaler Richtung. Dies widerspiegelt wohl die physiologische Funktion der endothelen Nierenzellen, von denen die MDCK Zellen abstammen, und legt die Vermutung nahe die Folge von endogen exprimierten Transportern unterschiedlichen Typs zu sein.

Im zweiten Ansatz testeten wir die Transporter-Hypothese der Pharmakoresistenz *in vivo*, in einem Medikamenten-resistenten Mausmodell der Temporallappenepilepsie. Dazu wurden wildtyp und knockout Mäuse, welchen die Gene für abcb1a fehlen, benutzt. Diese Mäuse wurden durch intrahippokampale Injektion von Kainat (KA) epileptisch gemacht. Nachdem intrahippokampale Elektroden implantiert worden waren, konnten die EEGs dieser Mäuse gemessen und analysiert werden. Der Effekt einer Injektion von unterschiedlichen Dosen von CBZ wurde mit dem Effekt des Trägerstoffs allein verglichen. Hierfür wurde die Anzahl und Dauer der Anfälle vor und nach den Injektionen analysiert. Alle Mauslinien zeigten drei Wochen nach der KA Injektion die charakteristischen Anfälle sowie eine hippokampale Sklerose. Wir fanden klare Unterschiede zwischen den verschiedenen getesteten Mausstämmen in der Anfallszahl, aber nicht in der Anfallslänge. Es schien auch so, als ob die Mäuse nach der Injektion von CBZ gehäuft interiktale Aktivität hätten. Nichtsdestotrotz konnte keine Beteiligung von Abcb1a an der CBZ-Resistenz nachgewiesen werden.

Mittels Kombination der beiden Studien sollte die Gültigkeit der Transporter-Hypothese für die Antiepileptika-Resistenz überprüft werden. Die Resultate stützen die Hypothese nicht, da *in vitro* keine Beteiligung der humanen Proteine ABCB1 und ABCC1 am Transport von Antiepileptika gezeigt werden konnte, noch konnte *in vivo* bewiesen werden, dass das Maus-Homologon Abcb1a eine Rolle bei der Resistenz spielt. Zusammen mit ähnlichen Befunden von anderen Forschungsgruppen stellen diese Resultate die Relevanz der Transporter-Hypothese in Frage. Man muss annehmen, dass nicht nur ein Mechanismus für die Antiepileptika-Resistenz in Patienten verantwortlich ist, sondern dass verschiedene zusammenspielen, einschliesslich die Beseitigung von Medikamenten durch deren Abfuhr oder deren Metabolismus, sowie funktionale Veränderungen der Wirkungsorte.

2 Summary

Temporal Lobe Epilepsy (TLE) with hippocampal sclerosis is a common adult seizure disorder, with resistance to antiepileptic drugs (AED) being a common limiting factor of its treatment. Pharmacoresistance might be due to enhanced AED removal from epileptogenic tissue at the blood-brain barrier (BBB) through over-expression of multidrug transporters, especially of the ABC transporter family. Hence, evaluation of the potential of an AED to be a substrate for ABC transporters located at the BBB will be crucial to understand its brain distribution under normal and pathological conditions and for future development of drugs with improved brain penetration. This work investigated the possible role of multidrug transporters of the ABCB and ABCC family in AED resistance using an *in vitro* and an *in vivo* approach.

We have established a novel *in vitro* BBB model using inducible expression of the human ABC transporters ABCB1 and ABCC1 in stably transfected polarised epithelial cells (MDCK cells). These cells build tight monolayers, hence forming a selectively permeable barrier separating a basal from an apical compartment when grown on filter inserts in cell culture wells. Transcellular diffusion or transport, either facilitated or active, are required for a substance to cross from one compartment to the other. With this system, it is therefore possible to assess the substrate specificity of any recombinantly expressed transporter. Furthermore, inducible expression by doxycycline allows using non-induced cells as a control. Monoclonal lines, transfected with cDNA constructs, were selected using Western blotting and immunofluorescence to verify the level of overexpression and the correct subcellular localisation of the recombinant proteins. Transport was assessed by adding a compound of interest to one compartment and, after a certain incubation time at 37ºC, measuring its concentration in each compartment, as well as intracellularly by LC-MS/MS analysis. To distinguish between the action of the overexpressed transporter and endogenous transport mechanisms, experiments were performed in parallel with non-induced cells. In addition, control experiments were performed at 4ºC to inactivate energy-dependent processes. The two first-line AEDs carbamazepine (CBZ) and lamotrigine could not be demonstrated to be substrates of either ABCB1 or ABCC1 under the condition of our assay. This result led to the conclusion that both drugs are weak substrates at best, of these human ABC transporters. Surprisingly, we observed that all these drugs and other compounds tested were transported through the MDCK cells

in basolateral to apical direction in a manner that was not influenced by the presence of the inducible ABC transporters. This feature might reflect the physiological function of epithelial kidney cells and is probably due to the expression of various endogenous transporters of various types.

In a separate study, we tested the multidrug transporter hypothesis of pharmacoresistance *in vivo* in a mouse model of drug refractory TLE. We used wild type mice and knockout mice lacking the abcb1a gene, which were rendered epileptic using intrahippocampal kainate (KA) injection. Occurrence of chronic recurrent seizures was determined by intrahippocampal EEG recordings. The efficacy of CBZ for suppressing seizures was compared to vehicle injection, by quantifying the frequency and duration of seizures before and after injection. All mouse lines displayed characteristic epileptic seizures combined with hippocampal sclerosis three weeks after KA injection. We found a marked strain-difference in seizure frequency but not seizure duration in response to CBZ. In addition, increased interictal activity was observed after CBZ injection. Despite these effects, an involvement of Abcb1a in resistance to CBZ could not be demonstrated unambiguously.

By combining both approaches we aimed to assess the validity of the transporter hypothesis for AED resistance. Our results do not support the hypothesis, as no involvement in AED transport of human ABCB1 and ABCC1 was found *in vitro,* nor could the mouse homologue Abcb1a clearly be demonstrated to play a part in resistance to CBZ *in vivo*. Our results, together with comparable findings of other groups, questions the relevance of the multidrug transporter hypothesis. However, it is possible that multiple mechanisms, including extrusion and metabolism of drugs, and functional changes at drug targets, act together to result in drug resistance to a multitude of different AEDs.

3 General Introduction

3.1 Epilepsy

Epilepsy is a major neurological disorder which does not only affect humans, but is older than mankind. Not only the human brain, is complex enough to generate the typical synchronised discharges which underlie a seizure. Due to the generalised seizures which may be symptomatic and appear quite spectacular, people with epilepsies have often been stigmatised negatively or positively in history. In the ancient world people with epilepsy have been considered holy and the old Greeks even called it the "Sacred Disease". Hippocrates was the first to state that it was not more or less sacred than any disease, but has a natural cause lying in the brain (Hippocrates, ca 400 BC). In the mediaeval period, seizures were often interpreted as demonic possessions and treated with Exorcism by the Catholic Church or affected people were burnt as witches. Etymologically the word "seizure" derives from "seize", implying that a supernatural power has taken hold of the person.

Even though today we know that neither devil nor God have much to do with epilepsy, we are still dealing with the same symptoms. The underlying seizures are caused by electromagnetic discharges in the brain.

Epilepsy is a chronic and often progressive brain disorder and has a world prevalence of about 1 % (Shorvon, 1996, Browne, 2001). It is characterised by recurring, unpredictable seizures. These typically last seconds to minutes but can prolong to become a continuous status epilepticus. Often it can be controlled with medication and in many cases spontaneous remission occurs, however, there is no real cure (Cockerell et al., 1995). It should be understood as a group of syndromes rather than a single disorder. The mechanisms underlying epileptogenesis are not clear, though, seizure development involves synchronised, rhythmic firing of neurons. The driving forces, hypersynchronisation of the neuronal circuitry and a hyperexcitability of the neurons, can have numerous sources (see below).

3.1.1 Pathology

The causes for epilepsy are diverse and are classified into three groups. i) Symptomatic: caused by a known disorder of the central nervous system (CNS) including preceding brain damage through tumour, trauma or through drugs of abuse and toxins; Or related to parasitic diseases such as neurocysteriosis, schistotomiasis or malaria (Dua et al., 2006). ii) Idiopathic: with a genetic predisposition but without

a known CNS damage. iii) Cryptogenic: where the cause remains elusive. (Shorvon, 1996, Avanzini and Franceschetti, 2003, Steinlein, 2004, Dua et al., 2006) Nevertheless, the boundaries between these forms are not strict and sometimes difficult to define.

Several types of epilepsy are discerned. The classification can be done by cause, manifestation of seizures, localisation of origin of the seizures in the brain or by the trigger leading to a seizure. The seizure-types are also classified. The main division is between generalised seizures, which affect the whole brain, and partial seizures, where the source of the seizure can be localised to a certain brain area. Partial seizures are subdivided into complex and simple partial seizures, depending to what extent consciousness is affected. Simple partial seizures can also progress into complex partial seizures and may even evolve into so called secondary generalised seizures. Generalised seizures are characterised by synchronised activity of neurons from both hemispheres combined with a loss of consciousness. They are subdivided, depending on the effect on the body, into absence seizures also referred to as 'petit mal', myoclonic and clonic convulsive seizures, atonic seizures and tonic-clonic seizures, also known as 'grand mal', which is the most severe and palpable seizure type. (ILAE, 1981)

Among the various different kinds of epilepsy, temporal lobe epilepsy (TLE) is the one with the worst prognosis. It is a form of partial epilepsy and results in focal seizures. Typically, patients who develop TLE have experienced a febrile seizure during early childhood. Histological analyses of surgical specimens from patients with TLE have revealed common occurrence of hippocampal sclerosis (HS) and focal mass lesion. HS is characterised by a selective neuronal cell loss and gliosis in the cornu ammonis (CA) region 1 (CA1) and the hilus, occasional dispersion of the granule cell layer of the dentate gyrus (DG) (*Figure 3.1*) as well as synaptic reorganisation of the mossy fibres (mossy fibre sprouting) (Thom, 2004). Incomplete seizure control is a major problem with up to 80 % or more affected TLE patients and often resection of the epileptic focus is the only possible treatment (Mattson et al., 1996, Stephen et al., 2001, Wieser, 2004). It is debated whether the rearrangements in morphology and functionality, combined with astrogliosis may cause or contribute to drug refractoriness and persistence of seizures even after prolonged treatment.

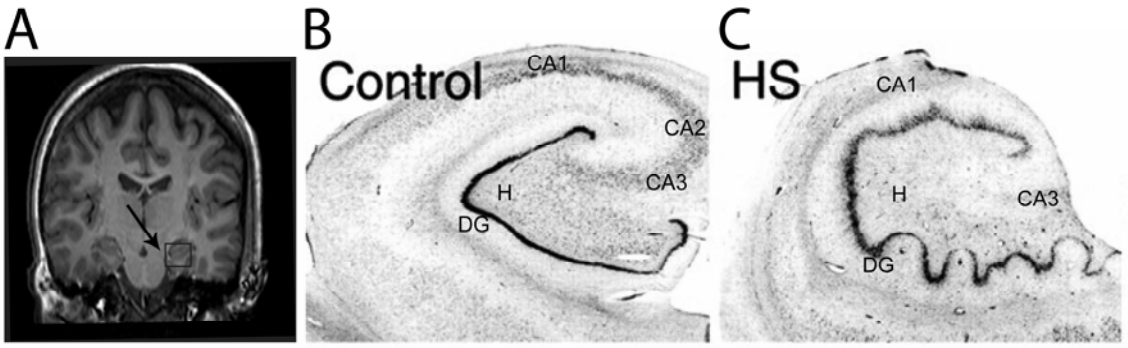


Figure 3.1: Human hippocampus, healthy and with TLE. *MRI overview of total healthy brain (C) and Nissl staining of human hippocampus cytoarchitecture from a control (autopsy) (A) and a TLE patient with HS (B). A: MRI with indication (arrow) of location of human hippocampus (box). B: Control, with a normal distribution of neuronal cell somata. C: TLE with HS, with prominent cell loss in CA1, CA3, and hilus (H) with relative sparing of the dentate gyrus (DG) granule cell layer and granule cell dispersion. The CA2 and part of CA3 pyramidal cell layer is not shown in this section. Adapted from (Loup et al., 2000).*

Seizures are in principal caused by hyperactive, synchronously firing neurons. The hyperactivity may be caused by a hyperexcitability of excitatory neurons. To generate action potentials, the ion distribution across the cell membrane and resulting transmembrane potential is crucial. If the balance is perturbed, e.g. through elevated extracellular K^+ levels in the resting state, neurons may depolarise easier, leading to hyperexcitability. Hence, any system required for the maintenance and reestablishment of the resting potential after a depolarisation may lead to seizure activity when impaired, suggesting a role in epilepsy. One example is the Na^+/K^+-ATPase, which, when blocked in hippocampal slices, can lead to epileptoform bursting activity in the CA1 region (Grisar et al., 1992, Vaillend et al., 2002).

Voltage-gated ion channels which are involved in the maintenance of the polarisation of the cell membrane are prone to play a role in epileptogenesis as well. Thus, if for example, voltage-gated Na^+ channels became activated at a more negative resting potential depolarisation would be facilitated which could potentially lead to a seizure. Indeed, various mutations in the subunits of voltage-gated ion channels have been linked to certain forms of epilepsy (Meisler et al., 2001, Avanzini and Franceschetti, 2003, Steinlein, 2004).

Another explanation for the development of seizures is a disturbance in the orchestration of inhibitory and excitatory synaptic transmission. To maintain proper brain function, the overall balance of excitatory and inhibitory neuronal activity must be maintained. Glutamate and acetylcholine (ACh) are excitatory neurotransmitters

whereas γ-amminobutyric acid (GABA) is the major inhibitory neurotransmitter of the CNS. Hyperexcitability may be caused by an imbalance of excitatory and inhibitory neurotransmitters, altered neurotransmitter signalling or a loss of inhibitory interneurons. GABA acts mainly via ligand-gated Cl^- channels, the pentameric $GABA_A$-receptors. Their activation leads to opening of the channels which in turn increases the membrane conductance due to possible Cl^- influx. This effect is known as shunting inhibition. Hence, a higher current is needed to change the membrane potential and to generate an action potential. In epilepsy, this inhibition may be lowered due to impaired GABA signalling by way of reduced levels of GABA or impaired sensitivity of the $GABA_A$-receptor to the transmitter. Loss of function mutations in $GABA_A$-receptor subunits as well as gain of function mutations in ACh-receptors have been found in heritable forms of epilepsy (Steinlein, 2004). Further it is known that excessive release of glutamate causes excitotoxicity and leads to neuronal cell death in status epilepticus (Fujikawa, 2005). Nevertheless, the relationship between neurotransmitters and seizures might not be so simple because of the complexity of neuronal networks with feedback and feedforward inhibition and due to changes in membrane potential gradients especially during development as reviewed in (Steinlein, 2004, Scharfman, 2007). Susceptibility for seizure development may also arise from effects of neuromodulators such as adenosine. By activating adenosine A_1 and A_{2A} receptors, high adenosine levels in the brain lead to tonic inhibition which can be reversed by receptor antagonists such as caffeine. Activation of the receptors by selective agonists or adenosine delivery by implanted cells have been shown to suppress seizures. On the other hand, overexpression of adenosine kinase, the main adenosine metabolising enzyme in the brain, has been found to contribute to epileptogenesis. Concluding, epilepsy may be associated with a dysfunction of the adenosine-mediated inhibitory tone (Boison, 2008).

3.1.2 Antiepileptic drugs

Treating epilepsy has always been a challenge. Non-pharmacological treatments include the ketogenic diet, various types of electrical stimulations like vagus nerve stimulation or deep brain stimulation and avoidance therapy, just to name a few. A vast number of treatments and drugs have been developed since the middle of the 19^{th} century when in 1857 the first effective drug was found: Potassium bromide. Bromides enhance the sensitivity of $GABA_A$ receptors to GABA and increase $GABA_A$ receptor currents as they are more sensitive to Br^- than to Cl^- (Gallagher et al., 1978).

Bromides are still in use for treatment of epilepsy in cats and dogs and were the best anticonvulsants until 1912, when the first barbiturate, phenobarbital was introduced. Phenobarbital has since been the most widely used antiepileptic drug (AED) and is also the oldest which is still commonly used especially in developing countries. Barbiturates enhance the effect of GABA by increasing the mean opening time of $GABA_A$-receptors, without affecting opening frequency or conductance but can also activate the receptor directly, in absence of GABA. This may also be the reason for the strong sedative effect of barbiturates (Kwan and Brodie, 2004).

In 1938, phenytoin started to replace the barbiturates as it does not display the sedative side effect. Phenytoin belongs to the class of hydantoins and reduces electrical conductance between neurons by stabilizing the inactive state of voltage-gated Na^+-channels. This action is believed to explain the protective action against generalised tonic-clonic as well as partial seizures (Francis and Burnham, 1992, Glauser, 2001).

Other AEDs acting on voltage-gated Na^+ or Ca^{2+}- channels include carbamazepine (CBZ) and its more recent, better tolerated derivative oxcarbazepine, of the carboxamides as well as the more recently introduced lamotrigine, a triazine. All three do also act as mood stabilisers and are used in treatment of bipolar disorders. CBZ and lamotrigine are still of the most widely used first line anticonvulsants used as monotherapy for simple and complex focal seizures as well as to treat generalised tonic-clonic seizures. Their modes of action are rather well understood. They stabilise the inactivated state of Na^+ channels (Schmutz et al., 1994, Kuo and Lu, 1997, Ambrosio et al., 2001). In 2000 the sulphonamide Zonisamide came into use which is also thought to act on Na^+- and Ca^{2+}- channels (Leppik, 2004).

Quite a few modern AEDs target the GABA system, just as the first drug, bromide, did. Commonly known are the benzodiazepines which act as CNS depressants. Their activity results from an allosteric modulation of $GABA_A$ receptors containing the γ2 subunit together with α1, α2, α3, or α5. For the anticonvulsant effect, receptor subtypes containing the α1γ2 subunit combination are necessary (Rudolph et al., 1999). Members of this drug family include diazepam, clonazepam or lorazepam (*Table 3-1*). However, due to strong side effects and dependence these drugs are mainly used for acute treatment of status epilepticus rather than long term prophylactic treatment.

Vigabatrin is a GABA analogon which acts on a GABA transaminase and thereby leads to elevation of GABA levels in the brain. However, the effect seems not to depend on

$GABA_A$ receptor-mediated synaptic response but on tonic inhibition which is probably due to higher extracellular GABA levels (Rogawski and Loscher, 2004). Another AED, tiagabin, a fatty acid, inhibits the GABA transporter GAT1 thereby slowing reuptake and clearance of GABA from the synaptic cleft and prolonging inhibitory postsynaptic potentials (Thompson and Gahwiler, 1992).

There are also a couple of drugs of with complex and/or mixed modes of action. E.g. valproate, also a fatty acid which was found to have anticonvulsant effects already in 1962, seems to have multiple modes of action including blockage of voltage-gated Na^+-channels and T-type Ca^{2+}-channels and also to act on GABA catabolism as GABA-transaminase inhibitor. These properties make valproic acid a broadband anticonvulsant drug.

For a number of drugs the mechanisms of action are still elusive or only poorly understood. For example levetiracetam, a pyrrolidine, seems to act on N-type Ca^{2+} channels, thereby inhibiting Ca^{2+} currents and has been suggested to bind and modulate SV2A, an integral membrane protein which has a crucial role in the regulation of vesicle function (Lynch et al., 2004, Rogawski and Loscher, 2004). Levetriacetam was approved as anticonvulsant in 1999 and is especially used as an adjunctive therapy in adult patients for whom current therapies have not been effective in controlling partial seizures.

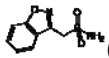
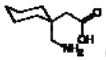
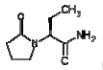
The fructose derivative topiramate seems to have a multitude of actions such as activation of $GABA_A$ receptors, action on kainate and AMPA receptors and blocker of Na^+ and Ca^{2+} channels (Privitera, 2001, Rogawski and Loscher, 2004).

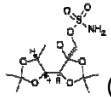
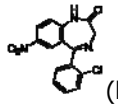
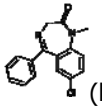
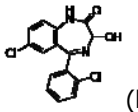
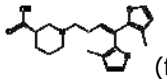
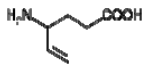
The GABA analogon gabapentin was designed to mimic the chemical structure of GABA. However, it lacks activity via $GABA_A$ receptors and its mode of action is poorly understood. Though, it binds to a subunit of high-voltage-gated Ca^{2+}-channels (Rogawski and Loscher, 2004).

Ethosuximide, a succinimide, is widely used in absence seizures (*Table 3-1*). There is some controversy over the exact mechanism by which it acts. However, it seems to be a T-type Ca^{2+}-channel blocker (Rogawski and Porter, 1990).

Research is still ongoing, ideally trying to find a cure for epilepsy, hence, taking in account genetic findings as well as various animal models of epilepsy. For future strategies of epileptic treatment, drug development is one possibility with the prospective that compounds with new modes of action will be developed.

Table 3-1: Antiepileptic drugs used to treat partial seizures.

Drug	Action on:				Protein binding (%)	F (%)	T_{max} (h)	Dose (mg/kg/d)	Chemical structure
	Na^+ channels	Ca^{2+} channels	GABAergic system	Glutamate receptors					
Predominant Na^+ (and Ca^{2+}) channel activity									
Carbamazepine	I_{NaF}				75	75 - 85	4 - 12	10 - 30	 (carboxamide)
Oxcarbazepine	I_{NaF}				45	> 90		15 - 30	 (carboxamide)
Lamotrigine	I_{NaF}	HVA			55	> 90	1 - 3	1 - 15	 (triazine)
Phenytoin	I_{NaF}, I_{NaP}				90	> 90	2 - 12	5 - 10	 (hydantoin)
Zonisamide	I_{NaF}	T-type			55		2 - 5	4 - 8	 (sulfonamide)
Mixed, complex or poorly understood actions									
Ethosuximide		T-type?			0	100	2 - 7	15 - 20	 (succinimide)
Gabapentin		HVA (α2δ)	↑ GABA turnover		0	30 - 60	2 - 3	30 - 40	 (GABA analogue)
Levetiracetam		HVA	reverses DMCM		≤ 10	100	1 - 2	15 - 40	 (pyrrolidine)
Phenobarbital		HVA	$GABA_AR$	AMPA	45	> 90	0.5 - 4	2 - 5	 (barbiturate)

Topiramate	I_{NaF}, I_{NaP}	HVA	$GABA_AR$	KA/AMPA	15	> 90	1 - 4	5 - 9	 (fructose derivative)
Valproate	I_{NaF}?, I_{NaP}?	T-type?	↑ GABA turnover		70 – 93[a]	> 90	1 – 8[b]	15 - 30	 (fatty acid)
GABA-mediated mechanisms									
Clonazepam			$GABA_AR$ allosteric modulation		85	≥ 90	1 - 3	0.02 – 0.2	 (benzodiazepine)
Diazepam			$GABA_AR$ allosteric modulation		95	> 90	1	0.2 – 0.5	 (benzodiazepine)
Lorazepam			$GABA_AR$ allosteric modulation		90	> 90	1.5 - 2	0.03	 (benzodiazepine)
Tiagabine			GABA transporter		96	> 90	1 - 2	0.1 - 1	 (fatty acid)
Vigabatrin			GABA transporter			80	0.5 - 2	40 - 100	 (fatty acid)

Adapted from (Bourgeois, 2001, Rogawski and Loscher, 2004), (Delanty, 2001) and (Bourgeois, 2001). AMPA, α-ammino-3-hydroxy-5-methyl-4-isoxazole-propionic acid; DMCM, methyl-6,7-dimethoxyl-4-ethyl-β-carboline-2-carboxylate (a negative allosteric modulator of $GABA_A$ receptors); F, bioavailability; GABA, γ-amminobutyric acid; $GABA_AR$, $GABA_A$ receptor; h, hours; HVA, high voltage activated; I_{NaF}, fast Na^+ current; I_{NaP}, persistent Na^+ currents; KA, kainate; NMDA, N-methyl-D-aspartate; protein binding, fraction bound to serum proteins, T_{max}, time interval ingestion to maximal serum concentration. [a]concentration dependent; [b]absorption of enteric coated tablets is delayed

3.1.3 Pharmacoresistance

Despite the vast number of drugs and combinations of drugs with various chemical and pharmacological properties available, some patients do not respond to treatment. In these cases surgical removal of the epileptic focus, if possible, remains the last possibility. Pharmacoresistance in epilepsy is not easy to define, however, an important characteristic is that many patients with refractory epilepsy do not respond to several drugs (Regesta and Tanganelli, 1999). Just as the disorder itself, the resistance to AEDs is probably multifactorial. Genetic factors may play a role, as well as disease related factors, such as aetiology, AED treatment or altered drug targets. On the other hand, the broad range of drugs with various modes of action argues against an epilepsy-induced alteration of specific targets but rather hints that non-specific, adaptive mechanisms are involved.

Three main hypotheses have been put forward so far: The change of drug target hypothesis, the enzymatic barrier hypothesis, and the multidrug transporter-mediated extrusion hypothesis. The first is characterised by reduced efficacy or affinity of an AED to its target. These include ion channels and neurotransmitter receptors, as well as enzymes involved in release, uptake and metabolism of neurotransmitters depending on the mode of action of AEDs (Schmidt and Loscher, 2005). One such mechanism found in animal models of epilepsy is channelopathy through a change of subunit composition in voltage gated ion channels. These are both, activated and inactivated by certain voltages. The current range between complete inactivation and the starting of activation is termed window current. Channelopathies may be leading to changes in Na^+-channel voltage dependence. Hence, channels open more readily resulting in an increased window current. Consequential, a persistently active Na^+ influx could amplify synaptic depolarisation (Ellerkmann et al., 2003, Schmidt and Loscher, 2005).

The enzyme hypothesis has recently been put forward. Briefly, it was found that endothelial cells of the blood-brain barrier (BBB) from patients with refractory epilepsy express more of the functional cytochrome P450 enzyme Cyp3A4 which can convert e.g. CBZ to its first metabolite 10,11-dihydrocarbamazepine (Janigro, 2008). Furthermore other metabolising enzymes such as epoxide hydrolases have been found to be active in the BBB and circumventricular organs (Ghersi-Egea et al., 1994). The resulting metabolism of AEDs would prevent them from reaching their target.

The third hypothesis, known from cancer research, is enhanced extrusion of drugs from the brain through multidrug transporters. These, located at the BBB, could be over-expressed or otherwise up-regulated in patients and prevent sufficient uptake of AEDs into the brain (Loscher and Potschka, 2002, Kubota et al., 2006). Multidrug transporters are expressed at the BBB (Kubota et al., 2006, Soontornmalai et al., 2006, Gazzin et al., 2008, Roberts et al., 2008) and their role in resistance is stressed by the fact that several studies claim to have found overexpression of multidrug transporters in epileptic brain tissue of patients and in epilepsy models (Tishler et al., 1995, Sisodiya et al., 1999, Zhang et al., 1999, Dombrowski et al., 2001, Sisodiya et al., 2001, Sisodiya et al., 2002, Aronica et al., 2003, Aronica et al., 2004, Volk et al., 2004). However, the data about transport substrates among AEDs is still scarce and controversial (Luer et al., 1999, Rizzi et al., 2002, Sills et al., 2002, Gibbs et al., 2004, Loscher, 2007, Luna-Tortos et al., 2008).

3.1.4 Animal models of epilepsy

To study epilepsy and AED action a multitude of acute and chronic models have been developed. Nevertheless, due to the fact that there are several types of epilepsies as mentioned above, it is not easy to come up with a relevant model for each form of the disorder. There are *in vitro* models, based on cell cultures, on rodent brain slices, on hippocampal slices or slice preparations from human brain specimens etc. On the other hand, there are a number of *in vivo* animal models for various types of epilepsy in various species including drosophila, zebrafish, various rodents, cats and monkeys. As variable as the species is the basis of the model: there are genetic (idiopathic) epilepsy models, and acquired (symptomatic) epilepsy models such as irradiation-induced models, electrical stimulation-induced models as well as chemically-induced models etc. These result in development of status epilepticus (SE), chronic seizures or acute and induced seizure activities in the animals (Pitkanen, 2006).

In this study, the focus lies on TLE. The three most commonly used chronic *in vivo* models for TLE in rodents are i) the kindling model, ii) the pilocarpine model and iii) the kainate (KA) model. Briefly, i) electrical kindling is usually done in rats. The animals receive a set of repetitive, short, sub-convulsive electrical stimuli in the hippocampus or the amygdala. This is repeated over several days until the seizure-threshold drops and the stimulations evoke seizures in the animals without strong morphological changes. This "kindled state" remains for the rest of the life of the

animals despite that no apparent changes occur. The animals may also have spontaneous seizures, after multiple days of triggering kindled seizures (Racine et al., 1975, McIntyre, 2006). However, this model is labour-intensive and does not go along with morphological changes, hence, it does not reflect the situation in human TLE patients very well. The absence of morphological changes in the hippocampus is also a weak point when one wants to study TLE with HS.

ii) The most commonly used chemically induced models of TLE are the pilocarpine and the KA model. TLE often occurs after an 'initial precipitating incident' which leads to the development of recurrant seizures. Such incidents include febrile seizures, trauma or SE. Both, pilocarpine and KA are convulsants and induce SE. Pilocarpine is an agonist of muscarinic acetylcholine receptors. When injected systemically in rodents, it induces acute seizures which lead to SE and later to chronic, spontaneously recurrent seizures and bilateral neuronal cell loss. As in patients which develop TLE after febrile seizures, the onset of chronic spontaneous seizures occurs only after a seizure free, latent period. However, the cell loss is severe and not restricted to the hippocampus but affects also other limbic regions and neocortical areas of both hemispheres. Besides, in the pilocarpine model, the animals develop only low seizure frequencies from 1 per day to 1 per month (Cavalheiro, 2006). This makes it hard to study e.g. drug effects on seizure development. Other problems are the high mortality due to the SE after injection of pilocarpine and that some individual animals do not respond and develop seizures at all.

iii) KA is an agonist of the kainate receptor, a glutamate receptor subtype. Depending on the route by which KA is administered, different subtypes of the KA model become manifest. If administered systemically, in a single, large dose it leads to a similar outcome as in the pilocarpine model with an induced SE followed by a latent period which is succeeded by a chronic phase; all going along with neuronal degeneration. In contrast to the pilocarpine model, the bilateral morphological changes are mainly found in the hippocampal formation and resemble strongly those found in TLE patients. The animals display spontaneouse nonconvulsive seizures, prolonged clusters of convulsive seizures, and can even develop spontaneous SE. In rats, seizures occur in frequencies between 0.1 – 0.6 per hour and last for an average of 1 to 3 minutes. However, comparable to the pilocarpine model, many animals die from the initial SE or, they do not experience SE at all, hence do not develop epilepsy (Dudek, 2006).

More recently it was established to inject KA intracranially e.g. into the hippocampus which does not cause as much damage as the systemic administration and leads to more reliable and reproducible results (Bouilleret et al., 1999, Dudek, 2006).

3.1.4.1 Kainic acid mouse model of temporal lobe epilepsy

As mentioned above, a mouse model of TLE with HS has been developed using unilateral intrahippocampal KA injection. This model can overcome the major disadvantages of the kindling model and the systemic pilocarpine and KA models (e.g. generalised seizures, bilateral cell damage, morphological changes in other brain regions, low seizure frequency).

Intracranial injection of a small dose of KA into the dorsal hippocampus induces non-convulsive SE that lasts for several hours. EEG recordings show synchronised, rhythmic, discharges in the ipsilateral hippocampus and cortex as well as contralaterally (*Figure 3.2 A, B*). KA injection initiates a slow, but progressive neurodegeneration which persists long after the drug has been cleared. Local lesions of the CA1 area, especially around the injection tract can be found at 24 h post injection. In the hilus and parts of the CA3 regions (also called CA3c) neuronal cell loss can be observed (Bouilleret et al., 1999, Riban et al., 2002).

After the SE the mice enter a latent phase which lasts for about 2 weeks. During this time, neurodegeneration of the ipsilateral CA1 and CA3c region as well as the hilus progresses, a large number of pyknotic cells can be seen. The granule cell layer of the dentate gyrus enlarges, the granule cells disperse (Bouilleret et al., 1999). EEG recordings in the ipsilateral side reveal a disappearance of normally occurring theta rhythm which gets replaced by a low voltage background activity with sporadic, isolated spike-and-waves (*Figure 3.2 C, D*). The contralateral side remains unchanged or with only minor alterations of theta activities (Riban et al., 2002, Arabadzisz et al., 2005).

Following the latent phase, mice begin to have frequent, recurring, spontaneous seizures. This marks the onset of the chronic phase which remains for the rest of their life. It is accompanied by further neurodegeneration which leads to a profound atrophy of the hippocampus (Bouilleret et al., 2000, Knuesel et al., 2001). EEG recordings show high voltage sharp waves lasting 4 to 10 s which are also seen in the contralateral hippocampus as well as typical hippocampal paroxysmal discharges lasting for 20 s or more (*Figure 3.2 E, F*). These are not seen contralaterally. Their

occurrence is highly variable within the recordings of a given animal but ranges from once or more every minute to once per hour or more (Riban et al., 2002, Arabadzisz et al., 2005). Morphologically the chronic phase is characterised by a complete degeneration of CA1 and CA3c pyramidal cells, extensive neuronal cell loss in the hilus a marked hypertrophy of the dentate gyrus with granule cells dispersed in a large part of the hippocampus (*Figure 3.3*). Additionally, mossy fibre sprouting in the supragranular molecular layer of the ipsilateral dentate gyrus and some of the contralateral was observed (Bouilleret et al., 1999).

The typical pharmacoresistance which makes treatment of patients with TLE often impossible is also a hallmark of this model (Riban et al., 2002, Gouder et al., 2003). This makes it a valuable tool to study the mechanisms of refractoriness to AEDs and to use it to find new approaches for treatments. To achieve this, mice can be chronically implanted with electrodes for EEG recordings. Thus, the seizure frequency can be surveyed under control conditions as well as conditions where animlas recive treatments. To study mechanisms of epileptogenesis, seizure suppression or drug resistance it is possible to use mice of various genotypes and with mutations in this model and to apply various compounds to see how they effect seizures (Riban et al., 2002, Gouder et al., 2003, Gouder et al., 2004, Fedele et al., 2005). With this kind of an approach new insights into the various aspects of epilepsy and its treatment can be obtained.

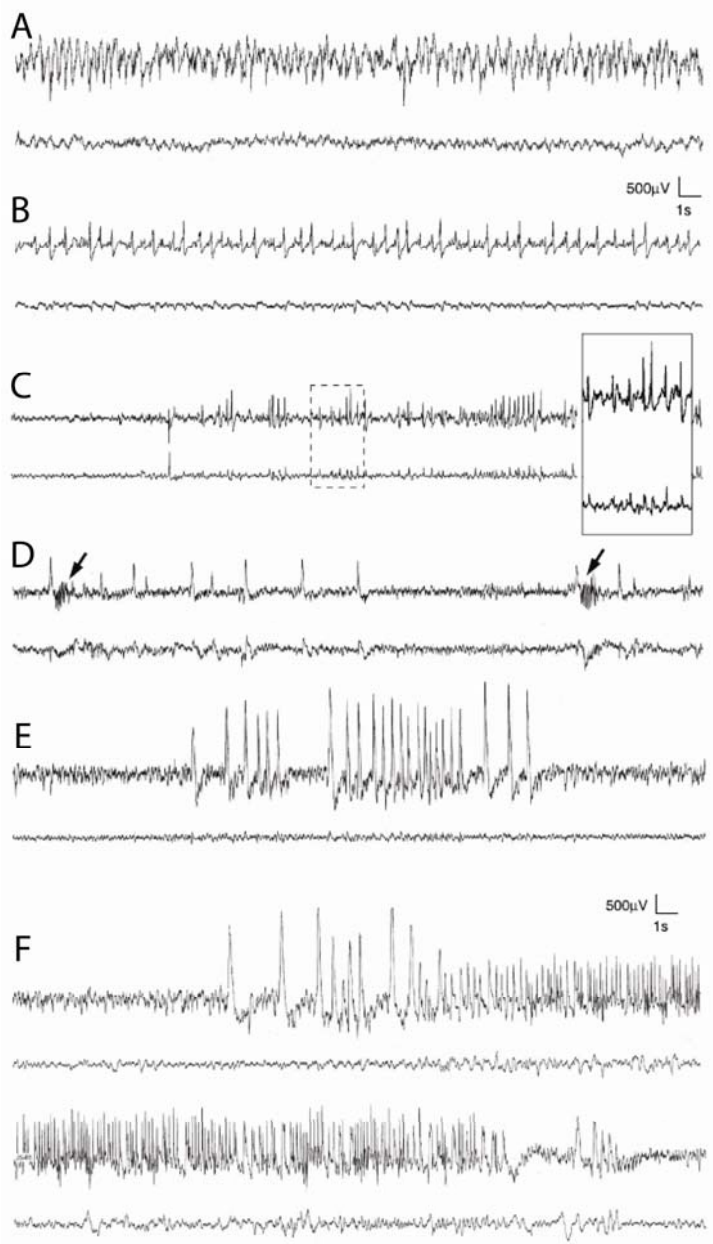


Figure 3.2: Typical EEG recordings of KA treated mice. *Upper traces: ipsilateral recordings with bipolar intrahippocampal electrodes; lower traces: ipsilateral recordings with monopolar intracortical electrodes. A: saline-treated mouse with normal theta activity. B: KA treated mouse during status epilepticus with continuous spikes or spikes and waves. C and D: Recordings during latent phase. C: background activity (left part of trace) and low voltage spikes, isolated or in groups. Enlargement shows propagation to the cortex which occurs sometimes. D: Bursts of high frequency activity (arrows) which occurs only at the end of the latent phase. E and F: Recordings during chronic phase. E: sharp waves. F: Hippocampal paroxysmal discharge, the typical seizure with high voltage sharp waves followed by higher frequency, low voltage rhythmic activity. Adapted from (Riban et al., 2002)*

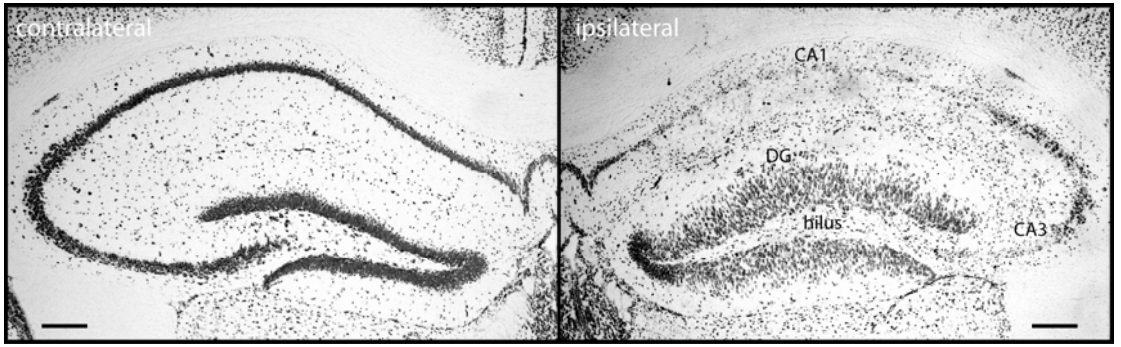


Figure 3.3: Nissl staining of mouse hippocampus cytoarchitecture after unilateral intrahippocampal injection of KA. *Contralateral: non-injected hippocampus displaying the normal morphology of the hippocampal formation. Ipsilateral: KA-treated side. Neuronal loss in hilus, CA1 and parts of CA3 (CA3c). Strong dispersion of dentate gyrus (DG) granule cells. Scale bar = 200 µm.*

3.2 ABC transporters and the blood-brain barrier

3.2.1 Blood-CSF and Blood-brain barrier

The brain has to be strongly protected against toxic agents and is thus shielded from the rest of the body by two barrier systems: the blood-brain barrier (BBB) and the blood-cerebrospinal fluid barrier (BCSFB). Their function is to protect the brain and cerebrospinal fluid (CSF) from toxic agents or antigens and at the same time ensure proper supply of the necessary nutrients or passage of hormones and growth factors. Basically they maintain the chemical composition of the neuronal "milieu" which is necessary for proper functioning of the brain. In many diseases, BBB breakdown is a major contributing factor e.g. in multiple sclerosis, brain tumour or HIV-associated dementia. Changes in BBB structure and composition have also been found in Alzheimer's or Parkinson's disease (Zlokovic, 2008).

The BCSFB is found in the ventricles, in a structure called choroid plexus (CP) which is responsible for the generation of the CSF. Each of the 4 ventricles has a CP consisting of a layer of epithelial cells which form villi, arranged around blood vessels on their basolateral side. Their apical side with a brush border membrane is facing the lumen of the ventricle. The epithelial cell layer is interconnected with tight junctions (TJ) which form the actual barrier. CSF is generated by filtering plasma from the blood through the epithelium. The process is driven by an osmotic gradient which is established by actively transporting ions into the ventricles. Water follows the gradient through pores formed by aquaporins in the membranes. Besides the ion-transporters, the CP is also equipped with a machinery to remove metabolic waste or xenobiotics from the CSF (Strazielle and Ghersi-Egea, 2000).

The BBB is built by the brain microvessel endothelial cells of the neurovascular system. With an estimated total length of more than 600 km and surface area of around 20 m^2, the capillary network in the brain forming the BBB is larger than the BCSFB and therefore potentially more important. Similar to the BCSFB, the actual barrier is generated by TJs that seal neighbouring endothelial cells together, hence, restricting the paracellular passage of molecules. TJs are complex structures consisting of a multitude of proteins such as occludin, several claudins, zonula occludens-1 (ZO-1), ZO-2, cadherins etc. These, or their adapter molecules, which link the TJ to the cytoskeleton, are often affected during chronic disease which may

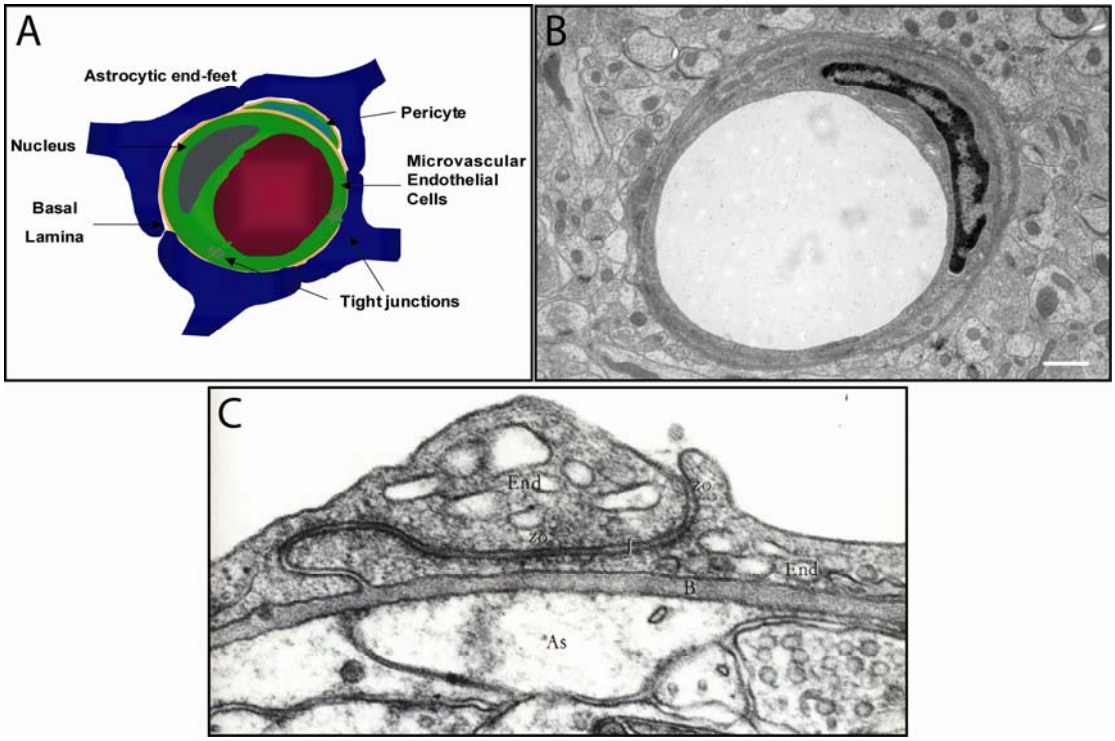


Figure 3.4: Schematic representation and electron micrograph of the BBB. *A: Schematic drawing of the BBB (Soontornmalai, 2006). Endothelial cells and pericytes are separated by a basal lamina. Astrocytic endfeet sheath the microvessel. B: Electron micrograph the cross section of a microvessel and surrounding brain tissue. Endothelial cell with nucleus and tight junctions between adjacent cells. Pericyte visible on the lower right hand side; scale bar 500 nm (picture kindly provided by C. Petitjean). C: close up of a tight junction (J) formed by two endothelial cells (End) (Alan Peters, 1991). The part where the membranes come closely together is called zonula occludens (ZO). The astrocytic endfeet (As) are separated from the endothelial cells by the basal lamina (B).*

lead to BBB leakage. The endothelial cells are surrounded by a basal lamina which separates them from their neighbouring cells, the pericytes and astrocytic endfeet (*Figure 3.4*). It is thought that the endothelial cells, the pericytes and astrocytes cooperate to build and maintain the basal lamina. The possible role of pericytes and astrocytic endfeet in the barrier function is still unclear.

The main barrier function is established by an extensive transport system regulating the transcellular passage of molecules. In general, 5 categories can be distinguished: i) carrier-mediated transport, ii) ion transport, iii) active efflux transport, iv) receptor-mediated transport and v) caveolae-mediated transport (summarised in *Figure 3.5*). In brief, i) carriers selectively facilitate diffusion of nutrients such as sugars, amino acids, nucleosides or vitamins. ii) Ion transporters are mainly ATP-driven pumps such

as the Na^+,K^+-ATPase which regulate the influx and efflux of ions to maintain appropriate concentration gradients. These gradients are required to drive e.g. Na^+ dependent transport. iii) active efflux is mainly initiated at the luminal membrane via ATP driven ABC transporters (see further down). iv) Transport of large proteins occurs mainly via receptor-mediated transport systems like the leptin BBB receptor. This system has also been targeted in pharmacological research for drug delivery via a strategy known as Trojan horses: agents are coupled to a monoclonal antibody against one of the BBB receptors (e.g. insulin). The antibodies act as surrogate ligands and can be used to shuttle the conjugated agent across the BBB via clathrin-dependent internalisation of vesicles containing the receptor. v) The caveolae control transcytosis in sphingolipid-rich microdomains (lipid rafts) of the BBB containing caveolin-1. The caveolar membranes contain specific receptors. Clathrin-independent endocytosis of these receptors is raft-dependent (Zlokovic, 2008).

Another basis for the barrier function is via metabolising enzymes. Endothelial cells express a large number of enzymes, including peptidases, esterases or drug-metabolising enzymes like epoxide hydrolases (Ghersi-Egea et al., 1994, Zolnerciks et al., 2007). These can modify molecules, which could otherwise bypass the physical barrier (Zolnerciks et al., 2007). Therefore they contribute to regulate the inner milieu of the brain.

3.2.2 ABC transporters

ATP-binding cassette transporters (ABC transporter) represent a protein superfamily, which is one of the largest, and most ancient families with representatives in all kingdoms from bacteria to animals. ABC transporters are energy-dependent integral membrane proteins which can carry a variety of diverse substrates across lipid bilayers by hydrolysing ATP (Hollenstein et al., 2007). Proteins are classified as ABC transporters based on the sequence and organisation. The basic domain organisation of ABC transporters consist of two transmembrane domains (TMDs), providing a passageway for the cargo, and two conserved cytoplasmic ATP-binding (and hydrolysing) domain(s), known as nucleotide-binding domains (NBDs). The NBDs are the motor domains of the transporters and consist of several conserved sequence motifs. The most important ones are the 'P-loop' or 'Walker-A' motif which binds the nucleotide; the 'LSGGQ' motif, also known as 'C-loop' or 'ABC signature motif' which contacts the nucleotide in the ATP-bound state; the 'Walker-B' motif that provides a

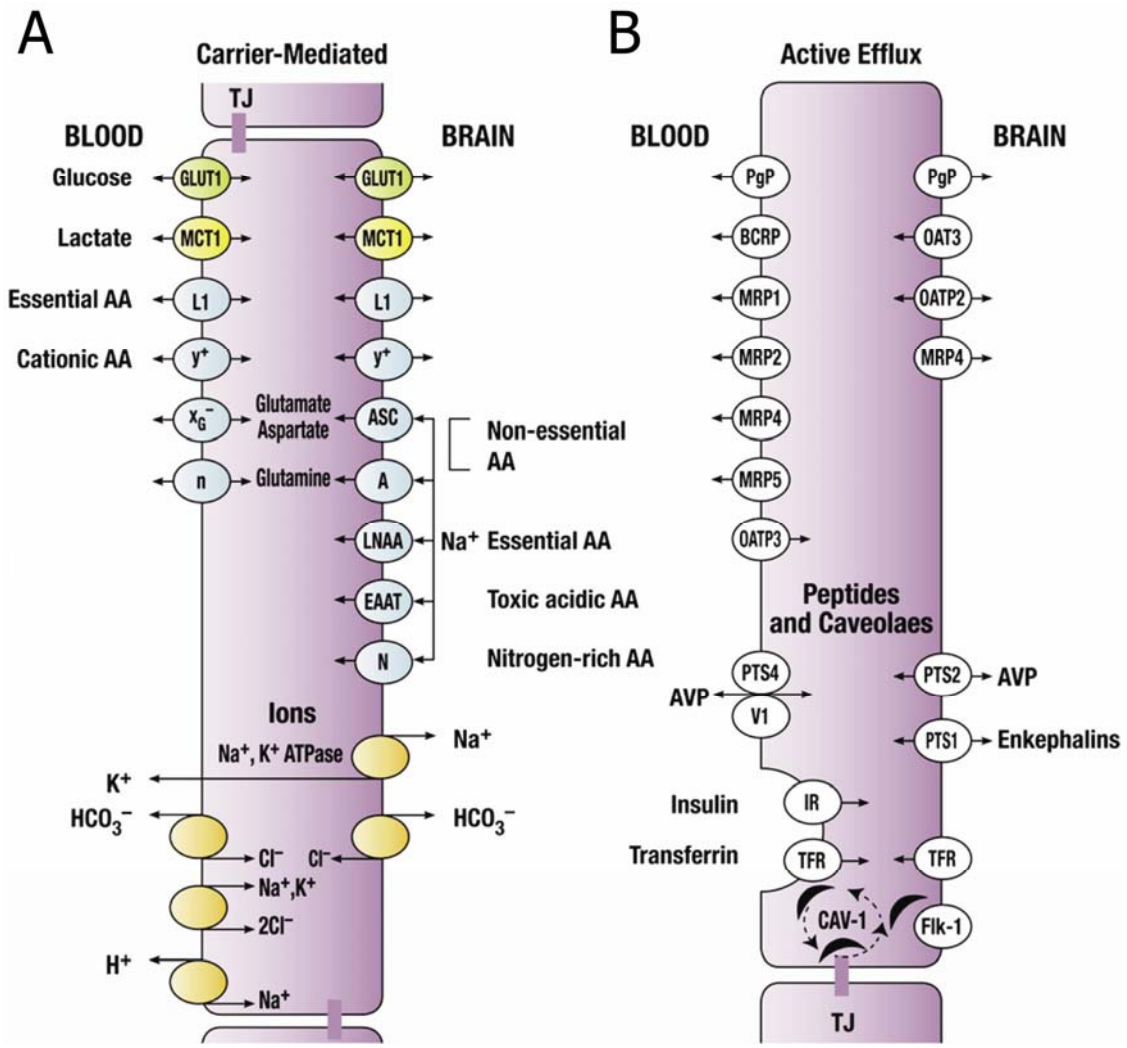


Figure 3.5: Simplified molecular atlas of the BBB: *A: Carrier-mediated transporters and their localisation on the luminal (blood) of the abluminal (brain-)side of the endothelial cells. Directions of transport are indicated with arrows. B: Active efflux transporters which are energy dependent. Direction of transport is indicated with arrows. Adapted from (Zlokovic, 2008).*

conserved glutamate residue important in ATP hydrolysis and a few more involved in subdomain interaction and the catalytic reaction (Hollenstein et al., 2007). Unlike the NBDs, the membrane spanning TMDs vary in primary sequence, length and architecture. Also the number of transmembrane (TM) helices varies between subfamilies. The two main subtypes are the ABC importers and the ABC exporters. ABC importers are present only in prokaryotes. In bacteria, exporters consist of two 'half transporters', each consisting of a TMD fused to a NBD. These form homo- or heterodimers to generate the functional unit. Many eukaryotic ABC exporters on the

other side are expressed with all four functional domains in one polypeptide chain (Hollenstein et al., 2007).

So far only crystal structures of prokaryotic ABC transporters have been solved, however, due to the strong conservation across species, these reveal possible mechanisms which apply to eukaryotic proteins as well.

3.2.2.1 ABCB1 and ABCC1

A subset of ABC transporters have been linked to transport of drugs and drug resistance. Among the mammalian ones a few have been demonstrated to have a well defined role in the transport of clinically relevant drugs. Hence, they are called multidrug transporters. The most important ones belong to the ABCB and ABCC, as well as the ABCG (BCRP or ABCG2) subfamily of ABC transporters.

Structure and function of ABCB1

The multidrug resistance protein 1 (ABCB1, also known as MDR1 or P-gp) of the ABCB subfamily was the first drug efflux pump to be discovered (Juliano and Ling, 1976). It consists of two similar halves, each containing an intracellular NBD and 6 putative TM segments (*Figure 3.6*). It is possibly the best studied ABC efflux transporter to date. Among many other substances, many anticancer drugs have been shown to be substrates of ABCB1. It was first identified due to its overexpression in cell lines, resistant to such cytotoxic drugs. There are a few common characteristics of multidrug transporter substrates. They are usually planar, heterocyclic, lipophilic organic compounds with a molecular mass ranging from less than 200 Da to about 800 Da; they often contain aromatic groups. The most efficiently transported molecules are uncharged or weakly cationic (Schinkel and Jonker, 2003, Higgins, 2007).

Crystal structure analysis mimicking the ATP bound state of the homodimeric *Staphylococcus aureus* multidrug transporter Sav1866, which is structurally related to ABCB1, revealed an outward facing conformation with the two NBDs in close contact and the two TMDs forming a central cavity (*Figure 3.6 B, C*). In this state the substrate could be released into the extracellular space. Hence, the simplest transport mechanism would involve two states: an inward-facing conformation with the substrate binding site accessible from the cytoplasm and an outward-facing conformation with an extrusion pocket exposed to the external medium. ATP hydrolysis is expected to return the transporter to an inward-facing conformation.

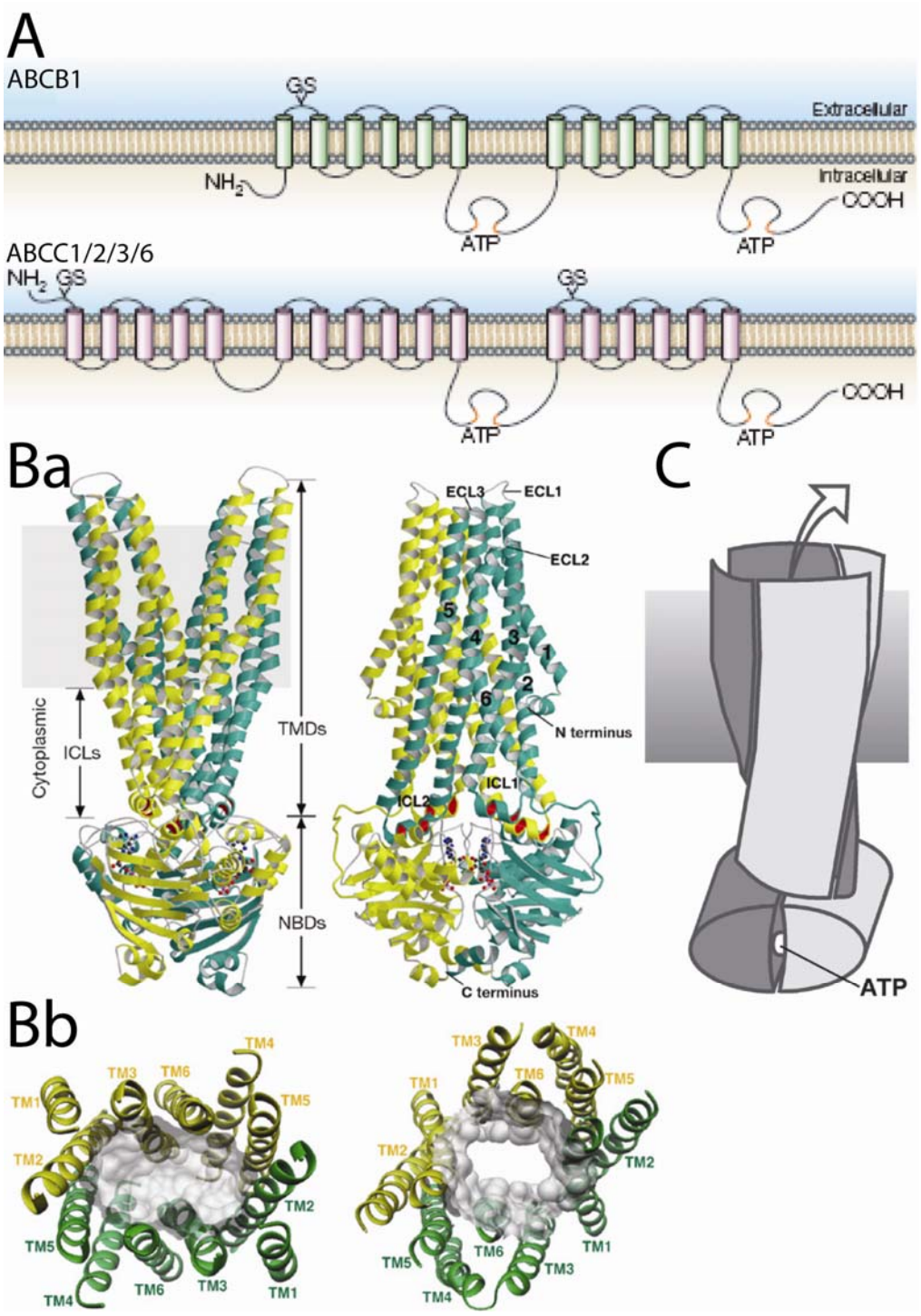


Figure 3.6: Structure of the ABC transporter families ABCB and ABCC. *A: Two-dimensional structural and topological models of brain multidrug transporters with transmembrane segments, glycosylation sites (GS) and NBDs (ATP). Compared to the ABCB1 and some members of the ABCC family, ABCC1, 2, 3 and 6 have an additional amino (N)-terminal domain, with five transmembrane segments. Except for the location of their glycosylation sites, their structure is similar to that of ABCB1. (Loscher and Potschka, 2005) B: Crystal structure analysis derived representation of the backbone of the homodimeric ABCB1 homologue Sav1866 in ribbon representation, with the subunits coloured yellow and turquoise.*

What was new from this structure analysis was the insight that the TMDs of Sav1866 from one subunit of the homodimer reach across and contact primarily the NBD of the opposite subunit and vice versa. Such swapping had not been anticipated for ABC exporters, but was not inconsistent with the available biochemical or genetic data. However, it contrasted the earlier idea of a side side-by-side arrangement of the two halves (Dawson and Locher, 2006).

From this and other recent crystal structures of full transporters it was postulated that ATP binding and hydrolysis by the NBDs drives inward-facing or outward-facing conformations of the TMDs. The key to effective unidirectional transport seems to be the ATP-driven 'closing' of the NBDs. Thereby the distance between the coupling helices decreases which in turn triggers a flipping of the TMDs from an outward to an inward-facing conformation. ABC exporters my now extrude bound drugs to the environment. Upon releasing ADP and inorganic phosphate, the transporter may flip back to an inward-facing conformation and exporters may recruit new substrates into high affinity binding sites. This *modus operandi* seems to be common for all types of ABC transporters (Hollenstein et al., 2007).

a: Bound ADP is in ball-and-stick representation. The view reveals the membrane-embedded 'wings' of the protein with the grey box depicting the probable location of the lipid bilayer based on hydrophobicity of the protein surface. right: 90° rotation compared to left side. The TM helices of one subunit (turquoise) are numbered. ICL, intracellular loops (between transmembrane helices); ECL, extracellular loops (between transmembrane helices); Bb: Views from the extracellular side into the transporter cavity at two levels. The transmembrane helices are numbered and the cavity is shown as a grey surface. Left: at the level of the inner leaflet. Right: at the level of the outer leaflet. Note that owing to helix bending, different subsets of helices line the cavity (Dawson and Locher, 2006).

C: Schematic of Sav1866 in the observed, outward-facing conformation. The cartoon emphasises the domain swapping and subunit twisting. Arrows indicate the release of bound drug into the extracellular space (Dawson and Locher, 2006).

ABCB1 is expressed in the intestine as well as in various barriers in the body such as the blood-testis barrier, the blood-nerve barrier or the foetal-maternal barrier in the placenta and also in the BBB. It seems obvious that ABCB1 has evolved to protect the vulnerable parts of the body from damaging effects of xenotoxins which were taken up with the food or generated by pathogenic organisms in the intestine (Schinkel and Jonker, 2003). ABCB1 is also expressed in the kidney where its function is less clear. It is abundant in the apical (luminal) membrane of proximal tubules. One would expect it to have excretory function there despite this has not been proven to date (Schinkel and Jonker, 2003). In rodents, ABCB1 has two orthologues: Abcb1a and Abcb1b. They are expressed with complementary tissue distribution. In the rodent brain, Abcb1 has been shown to localise at the apical, luminal membrane of microvascular endothelial cells (Soontornmalai et al., 2006, Roberts et al., 2008). Data about the distribution in the BCSFB is conflicting, nevertheless, data from our group as well as others suggests that Abcb1 is not present in the CP ependyma of rodents (Soontornmalai et al., 2006, Roberts et al., 2008). As mentioned above, there are studies which found that the transporter is overexpressed in brain from patients with intractable TLE (Dombrowski et al., 2001, Sisodiya et al., 2002, Aronica et al., 2004) or in brains from rodent models of TLE (Rizzi et al., 2002, Volk et al., 2004).

The ABCC family; structure and function

The multidrug resistance-associated protein 1 (MRP1) or ABCC1 was the first member of the ABCC family which was cloned in 1992 from drug-selected human lung cancer cells (Cole et al., 1992). The cells from which it was cloned showed similar resistance properties as were known from cells expressing ABCB1. Nevertheless, analysis of the computer-predicted topology of the membrane spanning domains (MSDs) revealed unusual properties for an ABC transporter. Also the two putative NBDs, which are highly conserved in most ABC transporters, were relatively divergent and resembled more to those of a chloride channel (CFTR) rather than a drug pump. Though, gene transfer experiments revealed that ABCC1 could confer resistance to drugs from several natural drug product families including *Vinca* alkaloids. It was also found to confer resistance to heavy metal oxyanions such as sodium arsenate where ABCB1 has no effect. This lead to the idea to compare it to the sequence of the protozoan *Leishmania tarentolae* ABC transporter ltpgpA, which had been shown to increase resistance to arsenicals. The two transporters proved to be homologues of each other, rather than of ABCB1. Since then it has become obvious that the ABCC

subfamily is so far the largest. It comprises 10 MRPs (ABCC1-6 and ABCC10-13) as well as the CFTR (ABCC7) and the sulfonylurea receptors SUR1 (ABCC8) and SUR2A/B (ABCC9) (Deeley et al., 2006).

Based on the predicted topology, the ABCC proteins fall into two groups: the short and the long ABCC proteins. ABCC4, 5, 7, 11 and 13 have a "typical" ABC transporter structure with two MSDs, each with 6 TM helices, while the others (ABCC1, 2, 3, 6, 8, 9, 10 and 12) have an additional n-terminal region which is poorly conserved but contains 4 – 6 TM helices (*Figure 3.6 A*). Within the family, the NBDs are highly conserved and contain all functional motifs as described above. The additional MSD of ABCC1 has only recently been ascribed a function in retention in and recycling to the plasma membrane (Deeley et al., 2006).

ABCC1 functions mainly as a (co-)transporter of amphipathic organic anions. It can transport hydrophobic compounds that are conjugated to glutathione (GSH), glucuronic acid or to sulphate and the export has been shown to rely on the presence of GSH in the cytoplasm. It is therefore likely that it exports drugs such as vincristine by co-transport with reduced GSH (Schinkel and Jonker, 2003).

The protein is widely expressed in the body with high levels in lungs, testis, kidneys, skeletal and cardiac muscles and the placenta but seems absent form the adult human liver (Deeley et al., 2006). ABCC1 is predominantly expressed on the basolateral side of epithelial cells and its substrates are therefore transported to the basolateral side of the epithelia. It contributes to the protection of tissues witch are shielded from blood-borne toxins by epithelia that have the basolateral membrane facing the blood circulation as is also the case in the CP (Deeley et al., 2006).

In the brain the findings are conflicting. However, Abcc1 the rodent orthologues of ABCC1 was found at basolateral membranes of BBB endothelial cells by our group and others (Soontornmalai et al., 2006, Roberts et al., 2008). Nevertheless, it's function there remains elusive and proof for expression at the basolateral membrane of healthy human microvascular endothelial cells is lacking despite that it was found in brain tissue from patients with TLE (Aronica et al., 2004).

Its presence in the CP on the other hand has been well established. Abcc1 is localised on the basolateral membrane of ependymal cells where it acts to protect the brain from toxins (Lee et al., 2001, Soontornmalai et al., 2006, Gazzin et al., 2008, Roberts et al., 2008).

3.2.3 Models of the BBB

To date, quite a few BBB models have been established. Hence the BBB is studied using *in vivo* or *in vitro* models. Depending on the focus of investigations, be it to investigate the cellular and molecular composition of the BBB, or the permeability, the properties of the model systems are adjusted. Nontheless, the most important feature is the regulated, specific permeability of the barrier.

Studies in animals include carotid artery injection, *in situ* brain perfusion or intracerebral microdialysis as reviewed in (Nicolazzo et al., 2006). All three techniques are used to assess permeability, especially uptake through the BBB for certain compounds such as drugs. They will be described briefly in the following. The carotid artery single injection, also known as brain uptake index (BUI) can be used in high-throughput settings as it is fast and easy. In this assay a small volume of buffered solutions containing the compound of interest and a radiolabelled diffusible reference compound is rapidly injected into the carotid artery. The animal is decapitated 5 to 15 s after injection and the brain and solute are analysed to calculate the BUI. The disadvantage is that the capillary transit time is very short and that the drug might back-diffuse from the brain to the blood or that it might be metabolised. However, it is suited to evaluate a large number of drugs in a short time (Nicolazzo et al., 2006).

In situ brain perfusion, an extension of the carotid artery single injection, has been used since more than 50 years and the technique has been simplified through the years. In this method, the animal (mostly a rat) is anaesthetised and the perfusion catheter is placed in the external carotid artery and the other ipsilateral arteries are cut. Then a compound of interest and a reference compound are perfused for a certain time. Following the perfusion the animal is decapitated and the compound concentration is determined. The problem with this technique, and also with the single injection technique, is that a compound which is not within the capillary can be either in the brain parenchyma (transcytosis), as is assumed, or can be caught in the capillary, either bound to the endothelia or inside the endothelial cells (endocytosis) (Nicolazzo et al., 2006). Thus, results have to be evaluated with this caveat in mind.

Intracerebral microdialysis involves direct sampling of interstitial fluid. A dialysis fibre is implanted into the brain; hence, the concentration of a compound that has permeated into the brain upon oral, intravenous or subcutaneous administration can be monitored over time. The microdialysis probe consists of a semipermeable membrane and is perfused with a physiological solution so small compounds traverse

the membrane in a concentration driven manner. The major advantage of this method is that not many animals need to be killed to assess the pharmacokinetic profiles of compounds in the brain. It is also quite accurate as both, plasma and brain levels of the compound can be determined over time and the probe can be placed in any region of the brain. This may be useful if one wants to assess the specificity of a targeted compound. However, the disadvantages are that only very low concentrations of compound may be present in the dialysate which may be hard to measure. Also, by insertion of the dialysis probe, a chronic disruption of the BBB could result (Nicolazzo et al., 2006).

However, the easiest way to study the role of a certain protein is to investigate its role in knock-out (ko) models. In this regard, a few mouse strains, lacking certain transporters which are thought to play a role in the BBB permeability, have been generated. These can be used in combination with one of the methods mentioned above or in an animal model for a disease or disorder such as the KA induced model for TLE which was described above.

In contrast to the *in vivo* models, *in vitro* models can more easily be used for high-throughput screenings. The era of *in vitro* studies of the BBB was opened more than 30 years ago in 1973, when Joó and co-workers (Joo and Karnushina, 1973) successfully isolated viable microvessels. Subsequent experiments provided important data on brain endothelial receptors, transporters and signalling mechanisms. Five years later, in 1978, the first observations on brain microvessel endothelial cells in culture were published by Panula and co-workers (for review see (Deli et al., 2005). Since then, the simplest models are based on one cell type. Still commonly used are primary microvessel endothelial cells isolated from cow, pig, rat or mouse brains or even from human brain specimens. As the extracellular matrix is crucial, also in culturing brain endothelial cells, they are grown on collagen coated substrates which leads to a higher trans-endothelial electrical resistance (TEER). The TEER varies between species from which the cells were isolated and ranges for example in bovine brain endothelial cells from an average of 500 - 800 $\Omega*cm^2$ (Deli et al., 2005). First insights into the molecular composition and functional properties of brain endothelial cells were obtained by growing them in culture dishes. To assess their permeability they are often grown on filter inserts (*Figure 3.7 A*) to obtain a two-compartment system (Joo, 1992). These primary or low passage microvessel endothelial cells

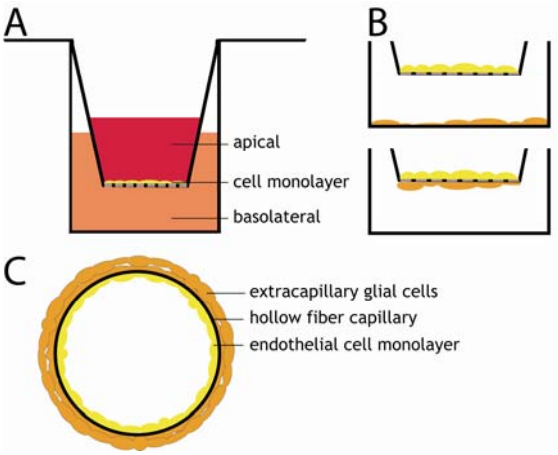


Figure 3.7: Schemes of in vitro models of the BBB. *A and B are static systems. A: tight monolayer of only one cell type (e.g. endothelial cells) dividing the system into two compartments: an apical (abluminal) and a basolateral (luminal) compartment. B: co-culture two compartment system. Top: non-contact method with co-cultured cells (e.g. astrocytes) growing on the bottom of the multiwell plate. Low: contact method with co-cultured cells growing on the underside of the filter. C: Dynamic system with endothelial cells monolayer growing on the inside of a porous hollow fibre capillary which is perfused with culture medium to provide shear stress. Glial cells grow in several layers on the outside of the capillary. The whole system is inside a sealed chamber (extraluminal space).*

provide closest resemblance to the *in vivo* BBB. However, the monocultures can only be cultivated for a limited number of passages before they lose their characteristic phenotype including the high TEER. By co-culturing astrocytes, which have also been shown to induce the development of TJ in brain endothelial cells, this de-differentiation can partially be prevented (*Figure 3.7 B*). Other neighbouring cells of the brain microvasculature have also been used in co-cultures. Among these are pericytes or even neurons (Deli et al., 2005). However, one has to keep in mind that for some applications the presence of other cell types might be a hindrance rather than an advantage as it might not always be possible to allocate an observed effect to one cell type and its components.

All the models mentioned above are static. Hence, they might not display the real situation which underlies also physical shear stress evoked by the blood flow. This lead the group around Janigro to develop a new, dynamic model which has a 3-dimensional tube structure with a continuous flow of culture medium, mimicking the physiological shear stress in blood vessels. While endothelial cells are grown in a monolayer inside the fibre capillary in a sealed chamber the medium can be pumped through the capillary in a continuous, pulsing flow, mimicking the blood flow in brain capillaries. On the outside of the porous capillary, glial cells, which are present around brain microvessels, are grown. These form several cell layers (*Figure 3.7 C*). This

model leads to very high TEER as found *in vivo* and seems well suitable to study transport properties (Stanness et al., 1997, Stanness et al., 1999).

In general, to appropriately mimic the BBB, there are some basic characteristics which a model must possess. These are: a) restricted paracellular transport which can be measured by the TEER. A minimal TEER of 150 – 200 $\Omega*cm^2$ was shown to be sufficient to assess drug permeability *in vitro*. However, that of the *in vivo* BBB is more than 2 $k\Omega*cm^2$. b) Brain endothelial cell characteristics including morphology (monolayer formation) and expression of the proper enzymes and BBB markers. c) Functional expression of BBB-specific transport machineries e.g. for nutrient transfer, efflux pumps etc. d) *In vivo*-like modulation properties such as alteration of permeability by interleukins, glial factors etc and e) Practicality of the system, including availability, convenience, predictability and reproducibility (Nicolazzo et al., 2006).

Primary cultures have disadvantages. They are expensive and time-consuming. Additionally, it is difficult to ensure purity and homogeneity of the cultures, since they are easily overgrown by contaminating cells such as pericytes (Deli et al., 2005). It is also difficult to genetically modify them by transfection due to their short life span.

To overcome some of these problems immortalised brain endothelial cells have been generated. However, none of the more than 20 cell lines can fulfil all the criteria which include the reproducibility and sufficient tightness due to incomplete formation of TJs which results in "leaky" barriers (Deli et al., 2005, Nicolazzo et al., 2006).

Due to the insufficient barrier properties of immortalised brain endothelial cell lines, it has become custom to use non-cerebral peripheral epithelial cell lines instead. One such line is the Madin-Darby canine kidney (MDCK) cell line. These cells are easy to grow and can be genetically modified by transfection. They are polar and upon transfection with a cDNA encoding a transporter such as ABCB1 will express it on the appropriate membrane (Pastan et al., 1988). They have also been shown to have high absorptive transport for CNS-positive drugs and low absorptive transport for CNS-negative drugs. Hence, they seem to be a suitable model for BBB permeation (Wang et al., 2005, Nicolazzo et al., 2006). On the other hand, MDCK cells are more cuboidal than brain endothelial cells and therefore have a relatively larger transverse area of intercellular TJs than brain endothelial cells. Consequently, paracellular transport may be overestimated with these cells. One also has to keep in mind that the expression pattern of transporters is not exactly the same in MDCK cells as in

brain endothelial cells. Caco-2 cells have also been used in BBB models. Like MDCK cells they are epithelial cells and have different morphological characteristics to brain endothelial cells (Nicolazzo et al., 2006). Common for all cell lines is that they are frequently used as BBB model when grown to tight monolayers in a two-compartment filter-insert system as shown in *Figure 3.7 A*.

3.2.4 Transport of AEDs by ABC transporters

So far, several groups have used transfected MDCK cells or other epithelial cell lines to study AED transport by recombinant ABC transporters. To date, most studies were performed in cells which constitutively express the recombinant proteins and non-transfected cells were used as background controls (Evers et al., 2000a, Evers et al., 2000b). Additionally, if one wants to study the role of an efflux transporter it is also possible to use cancer cell lines that are resitant to drugs through the expression of a specific transporter. These can be used in cytotoxicity assays and in uptake assays. In the cytotoxicity assay a substrate can be determined by addition of the compound of interest to the cells together with a cytotoxic agent to which the cells are resitant (e.g. vinblastine in case of ABCB1 expression). If the cells lose their resistance, the compound of interest is considered a substrate which is transported in a competitive manner. A similar phenomenon is tested in the uptake assay. Here cells are treated with the compound of interest during uptake of a radiolabled substrate. If the uptake is reduced, the compound of interest is considered a substrate as well (Rivers et al., 2008).

Despite, or because of the use of various *in vitro* and *in vivo* systems, transport data for ABC transporters are conflicting. Compounds which are good substrates such as vinblastine or vincristine could be shown to be transported with high transport ratios (TRs). These have since been used as positive controls in transport studies (Paul et al., 1996, Mao et al., 2000). Especially data about the transport of AEDs by the ABC transporters ABCB1 and ABCC1 are conflicting and for some drugs rather scarce. For others, like ABCC2, even less is known. To our knowledge only two *in vitro* studies on the transport of lamotrigine were carried out. Rivers et al. (2008) have not found it to be a substrate of either transporter in cytotoxicity and uptake assays whereas Luna-Tortos et al. (2008) found it to accumulate apically after prolonged incubation in a concentration equilibrium system expressing ABCB1. *In vivo* data on lamotigine transport in mice using blood-serum concentrations could not prove it to be a substrate of Abcb1a (Sills et al., 2002). A few more studies were performed on CBZ

transport by ABCB1 or ABCC1, nevertheless, data is controversial. While no transport by Abcb1a could be measured in mice *in vivo* by Owen et al. (2001a), Potschka et al. (2001) found it to be a substrate of both, ABCB1 and ABCC1 in a microdialysis study in rats. However, in subsequent cell culture studies by the same group, comparing transport by recombinant human and mouse proteins, this finding could not be verified (Baltes et al., 2006). Additionally, other groups could not find *in vitro* evidence for CBZ to be a substrate of ABCB1 or ABCC1 either (Owen et al., 2001a, Rivers et al., 2008). It was criticised that weak substrates might be missed due to masking of transport by passive diffusion in the commonly used *in vitro* studies. Most studies have been performed in concentration gradient setups where a compound to be tested is added to one side of a two-chamber system, hence passive, osmoses-driven diffusion could occur and mask active transport. To rule this out, Luna-Tortos et al. (2008) developed a concentration equilibrium system in which they studied transport of AEDs. With this they found in prolonged experimental setups that lamotrigine is transported by ABCB1 but CBZ is not. In case of ABCC2, a study in naive rats using microdialysis gave no evidence of its involvement in the entry of CBZ or lamotrigine into the CNS; but Abcc2 deficiency was associated with an increased anticonvulsant response to CBZ in kindled rats (Potschka et al., 2003). For the evaluation of this kind of data one has to keep in mind that there might be differences of substrate recognition of the transporters from different species. This is stressed by the finding that the AEDs phenytoin and levetiracetam were directionally transported by mouse but not human ABCB1, whereas CsA was transported by both types of ABCB1 (Baltes et al., 2006). A main caveat with all cell culture studies is the lack of a good baseline control. It is common practice to use non-transfected MDCK cells as baseline. However, these have been cultivated separately for some time and may have diverged, not featuring the same original characteristics any longer. MDCK cells have a wide range of endogenous transporters which may also contribute to drug transport. If the control cells do not express them to the same extent as the transfected cells, false positive results may be obtained or, as with passive diffusion, weak transport might be masked. This can partially be overcome by the use of inhibitors to potential endogenous transporters, however, these inhibitors are not always very selective and in some cases can also act as enhancers (Huisman et al., 2002, Zimmermann et al., 2008). Thus it is important to establish a good baseline control. In this work, we used MDCK cells with inducible expression of recombinant

transporters. Hence, cells cultivated together and from the same clone were used in transport studies when the transporter was induced, and served as baseline control in the non-induced state.

In vivo studies of AED transport have often been performed using microdialysis to analyse brain penetration of a certain drug. This can be tricky to analyse if the concentrations are low. In this study, we wanted to assess the involvement of ABC transporters in drug resistance by looking at the direct effect of an AED in epileptic mice which do or do not express the transporter of interest.

3.3 Aims

What is the role of multidrug transporters of the ABCB and ABCC family in AED resistance in TLE? This was the question we wanted to answer with this PhD thesis. It is thought that multidrug transporters of the ABC superfamily are responsible for multidrug resistance in TLE. However, the mechanism is not yet clear. So far, it was established that some ABC transporters are present at the BBB or BCSFB. However, unambiguous proof of their involvement in drug export, resulting in sub-threshold concentrations in the brain and target areas, has so far not been found. Also, it is not clear which AEDs are substrates for any of the ABC transporters present in these barriers. Hence we set out to assess substrate specificity for the two ABC transporters ABCB1 and ABCC1 using two approaches, one *in vitro* and one *in vivo*.

For the *in vitro* approach, we aimed to generate a barrier model which is better suited for the analysis of drug transport by a specific multidrug transporter than previously established models. To this end, we generated a novel BBB model, comprised of MDCK cells stably transfected with an inducible expression cassette containing either human ABCB1 or ABCC1 cDNA. The inducibility of the transporter expression should ensure to have a better baseline control for transport experiments than simply non-transfected MDCK cells. The baseline of active and passive transport should be the same in induced and non-induced cells, therefore, induced transporter-mediated effects should be clearly identifiable. With this system, we expected to be able to unambiguously assess whether two first line AEDs, CBZ and lamotrigine, are substrates of either of two ABC transporters, ABCB1 or ABCC1, which have been shown to be expressed at the BBB.

In the *in vivo* approach, we sought to test the multidrug transporter hypothesis in the intrahippocampal KA injection mouse model of TLE. Mice in this model had previously been shown to be pharmacoresistant to several AEDs (Riban et al., 2002, Gouder et al., 2003). We used wild type (WT) and knockout mice lacking the abcb1a gene. We wanted to test if the mouse ABC transporter is responsible for this drug-resistance. Hence, the effect of the AED CBZ was assessed in this model by analysing seizure frequency and duration before and after injection of the drug or vehicle in WT and in transporter-deficient mice. If Abcb1a were responsible for drug resistance in this model, a marked reduction of seizures in the transporter-deficient mice compared to WT would be expected. Previous experiments have shown that, under physiological conditions, the ABC transporters Abcb1a and Abcc1 do not influence the CBZ-induced

sedation and thus, do not seem to mediate lowered brain penetration of small amounts of CBZ in healthy mice (Soontornmalai, 2006). Thus, we wanted to assess whether the role of the ABC transporter is different in epileptic mice and with higher doses of CBZ than with lower doses in healthy mice.

By combining results from both approaches we aimed to assess the validity of the transporter hypothesis for AED resistance.

4 Results: *in vitro* study

Establishment of a blood-brain barrier model using inducible expression of ABCB1 or ABCC1 in MDCK cells to assess their involvement in antiepileptic drug resistance

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Submitted

4.1 Abstract

Pharmacoresistance in neurological disorders such as epilepsy might be due to insufficient drug levels in the brain through overexpression of multidrug exporters of the ATP-binding cassette (ABC) protein superfamily at the blood-brain barrier (BBB). Determination of the substrate specificity of ABC transporters for antiepileptic drugs (AED) will be crucial to understand their brain distribution under normal and pathological conditions and for future development of drugs with improved brain penetration. Here, we characterised a novel *in vitro* BBB model to assess the substrate specificity of the human ABC transporters ABCB1 and ABCC1 for carbamazepine and lamotrigine, two first-line AED. We established monoclonal MDCK cell lines, stably transfected with inducible expression-cassettes for ABCB1 and ABCC1, respectively. Immunocytochemistry and immunoblot analysis revealed correct subcellular localisation of recombinant protein in polarised cells after doxycycline-mediated induction of expression; formation of functional tight junctions could be confirmed by measuring transepithelial electrical resistance. To assess drug transport we measured changes in drug concentration by LC-MS/MS analysis in the basolateral and apical compartment of a two-compartment system, as well as intracellularly. Genetically identical, non-induced cells served as baseline control. The functionality was demonstrated using vinblastine and vincristine, substrates of ABCB1 and ABCC1 respectively. Surprisingly, we observed that all compounds tested are transported in a direction-specific manner through MDCK cells, independent of the presence of inducible ABC transporters. This may be due to the expression of potent endogenous transporters. Despite the operational system, no evidence was found that carbamazepine or lamotrigine are substrates of either ABCB1 or ABCC1.

4.2 Introduction

Epilepsy is a common neurological disorder affecting 0.5 - 1.0% of people worldwide. Despite the availability of several first-line antiepileptic drugs (AEDs) with different modes of action, including carbamazepine (CBZ), phenytoin, and lamotrigine (French et al., 2004, Marson et al., 2007), pharmacoresistance remains a major challenge in the clinical management of epilepsy, affecting a large fraction of patients with temporal lobe epilepsy (Stephen et al., 2001).

The biological basis of pharmacoresistance is unclear but likely to be multifactorial and can arise as a consequence of epilepsy (Jandova et al., 2006), or can be pre-existent, for example due to a modified drug target. For AEDs interacting with neurotransmitter receptors or ion channels, alterations in channel structure or accessory proteins leading to reduction of drug affinity or efficacy have been postulated (Ellerkmann et al., 2003, Schmidt and Loscher, 2005). Pharmacoresistance might also reflect impaired penetration of AEDs into the brain, due to overexpression of transporters of the ATP-binding cassette (ABC) superfamily at the blood-brain barrier (BBB). These may prevent therapeutic concentrations of AEDs in the epileptic focus despite adequate plasma levels (Loscher and Potschka, 2002, Sisodiya, 2003). Increased expression of ABC transporters in tissue from patients with drug-resistant epilepsy (e.g. (Dombrowski et al., 2001, Sisodiya et al., 2002) and seizure-induced expression in animal models (Zhang et al., 1999, Kwan et al., 2002, Volk et al., 2004) support this theory.

The best characterised ABC transporters are the "multidrug resistance protein" ABCB1 (MDR1 or P-gp; in rodents Abcb1a and Abcb1b) and "multidrug resistance-associated protein 1" ABCC1 (MRP1; in rodents Abcc1). ABCB1 is highly abundant in organs with secretary function and blood-tissue barriers like the BBB, where it resides at the luminal (apical) side of capillary endothelial cells (Soontornmalai et al., 2006, Roberts et al., 2008). ABCB1-immunoreactivity has been reported in neurons and glial cells in epileptic tissue (Dombrowski et al., 2001, Sisodiya et al., 2002, Aronica et al., 2004). It extrudes a range of structurally and functionally unrelated substances (Kwan and Brodie, 2005). Data for AEDs is controversial. For example, CBZ was not found to be an ABCB1 substrate *in vitro* and in Abcb1a/1b-null mice (Owen et al., 2001b, Baltes et al., 2006). In contrast, increased CBZ levels were found by microdialysis in rat brain when Abcb1 was blocked by verapamil infusion (Potschka et al., 2001). Finally,

directional *in vitro* transport of lamotrigine by ABCB1 has been described (Luna-Tortos et al., 2008).

ABCC1 is highly expressed in lungs, testis, kidneys and choroid plexus (CP) (Deeley et al., 2006). Its distribution in brain, which might be altered by certain pathologies, is not yet firmly established. In addition to microvasculature, parenchymal expression in epileptic foci was suggested (Sisodiya et al., 2002, Aronica et al., 2004). Abcc1 was detected immunohistochemically basolaterally in mouse brain endothelial cells (Soontornmalai et al., 2006, Roberts et al., 2008). A recent study in rats found Abcc1 predominantly expressed in the CP rather than the microvascular endothelium (Gazzin et al., 2008). Like ABCB1, ABCC1 has a wide substrate range, mostly organic anions, which are transported either when conjugated to glutathione (GSH), glucuronide or sulphate, or unmodified with GSH co-transport (Leslie et al., 2001). Data for AEDs as substrates of ABCC1 is inconclusive. While increased cerebral CBZ levels were found by microdialysis in rat brain when Abcc1 and Abcc2 were blocked with probenecid (Potschka et al., 2001), recent *in vitro* data argue that ABCC1 is no transporter for several AEDs, including CBZ and lamotrigine (Rivers et al., 2008).

In vitro drug transport assays across a monolayer of epithelial cells overexpressing an ABC transporter represent a substitute for the BBB (Baltes et al., 2006). However, such systems are limited by potential loss of transgene expression over time, difficulty to assess expression levels of recombinant proteins, and lack of suitable control for endogenous transport mechanisms. The aim of the present study was to establish an *in vitro* assay based on inducible expression of ABC transporters (ABCB1 or ABCC1 and eGFP reporter) in monoclonal lines of Madin-Darby canine kidney (MDCK) cells. This cell line was selected for its capacity to form tight monolayers with polarised membrane protein distribution. Inducible expression was driven by a tetracycline-regulated bidirectional promoter, allowing direct comparison of the drug transport capacity of genetically identical cells in the absence (non-induced, vehicle-treated cells) or presence (induced; doxycycline-treated cells) of the recombinant transporter to be tested. Upon morphological and functional characterisation of monoclonally selected cell lines, two first-line AED, CBZ and lamotrigine, were tested as potential substrates of ABCB1 and ABCC1.

4.3 Material and Methods

4.3.1 Plasmids

The antibiotic resistance cassette (SV40-zeocin) of the commercial vector pBudCE4.1 (Invitrogen, Basle, Switzerland) was replaced by the β-lactamase/SV40-neomycin/kanamycin resistance cassette (pBUDneo/kan). The gene for the reverse tetracycline-dependent transactivator (rtTA), derived from plasmid pUHrt 62-1 (kind gift from Prof. H. Bujard, ZMBH Heidelberg, Germany), was generated by PCR to destroy an internal 3' XbaI site and ligated into pBUDneo/kan (KpnI/BclI) 3' of the promoter for human elongation factor 1α (pBUDrtTA-XbaI). A TetO7 responder cassette, consisting of seven Tet-operator sequences flanked by two, bi-directionally pointing CMV minimal promoters and an eGFP gene from pGFPlacZ (Krestel et al., 2001) on one arm was cloned into pBUDrtTA-XbaI (XbaI/SpeI) resulting in plasmid prtTA-eGFP-TetO. The cDNA of human ABCB1 (wt haplotype C1236-G2677-C3435) from pGEM3Zf(-)Xba-ABCB1.1 (LGC Promochem/ATCC, Molsheim, France) was then cloned into prtTA-eGFP-TetO (XbaI) resulting in prtTA-eGFP-TetO-ABCB1.

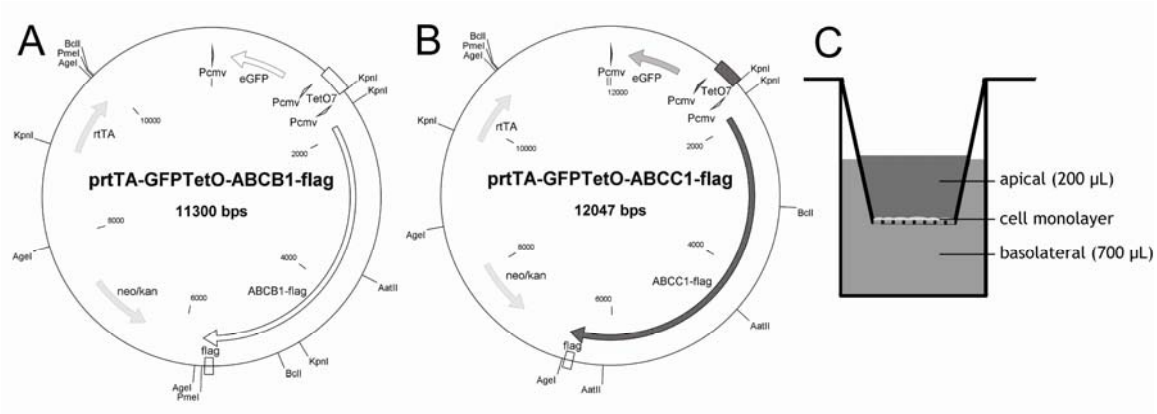

***Figure 4.1: A** and **B:** Vector maps of the two generated plasmids indicating the main restriction sites used during the cloning procedure, as well as the relevant promoters and genes used in this study. **A:** Vector containing flag-tagged ABCB1 cDNA (black boxed arrow) and bi-directionally expressed eGFP (grey boxed arrow). **B:** vector containing flag-tagged ABCC1 cDNA (black arrow) and bi-directionally expressed eGFP (dark grey arrow). **C:** Schematic drawing of in vitro BBB model. MDCK cells build a tight monolayer on "Transwell" membrane inserts dividing the well in an apical and basolateral compartment.*

Full-length human ABCC1 cDNA (non-synonymous SNP T117M, synonymous SNP N293N, L560L; kind gift of Prof. P. Borst, The Netherlands Cancer Institute, Amsterdam, The Netherlands) from pRC/RSV (Zaman et al., 1994) was cloned into prtTA-GFPTetO (blunt end) to result in rtTA-GFPTetO-ABCC1.

The two plasmids were re-cloned to contain a 3' triple flag-tag for better detection in immunocytochemical and immunoblot assays of ABC-transporter proteins. PCR with primers containing a triple flag-tag was used to amplify the 3' end of the respective ABC transporter gene which was then cloned back into the plasmids (PmeI partial/AatII for rtTA-eGFP-TetO-ABCB1-flag; AatII/AgeI partial for rtTA-GFPTetO-ABCC1-flag) (*Figure 4.1 A and B*).

4.3.2 Generation of stably transfected MDCK cell lines

Plasmids prtTA-eGFP-TetO-ABCB1-flag and -ABCC1-flag were linearised (PvuI) and MDCK cells were transfected (LGC Promochem/ATCC) using the cell line nucleofector kit L (for MDCK cells; Amaxa, Cologne, Germany). Cells were grown on TPP® (TPP, Trasadingen, Switzerland) plastic culture dishes in MEM supplemented with 10% v/v foetal calf serum, 1% v/v L-glutamine, 1% v/v non-essential amino acids (all from Gibco, Invitrogen), 1% v/v sodium pyruvate (Sigma-Aldrich, Buchs, Switzerland). Stable genomic integration was selected for by addition of 400 µg/mL G418 (Sigma-Aldrich). After about 14 days, when all cells in a mock-transfected culture were dead, the selection was considered to be complete. G418 was always added to maintenance cultures but not for experimental cultures. Monoclonal cell lines containing prtTA-GFPTetO-ABCB1-flag (thereafter referred to as MDCK-ABCB1) or prtTA-GFPTetO-ABCC1-flag (referred to as MDCK-ABCC1) were generated by limited dilution in selective maintenance medium and subsequent picking of cell clones using cloning cylinders (Scienceware, Sigma-Aldrich). These clones were expanded and analysed by Western blotting and immunocytochemistry (ICC) to verify inducible expression of the gene with 0.5 to 1 µg/mL doxycycline hyclate (DOX) (Sigma-Aldrich) and correct targeting of the protein to the apical or basolateral cell surface. Monoclonal lines fulfilling all requirements were chosen for further experiments. The cell lines were used for transport experiments for up to 7 passages after transfection.

4.3.3 Immunoblot

Two µg of protein from cell extracts of induced (DOX-treated for 24 h) or non-induced (non-treated with DOX) cells were analysed using a 7.5% SDS-PAGE and subsequent transfer onto nitrocellulose membranes (Osmonics, EGT Chemie, Taegerig,

Switzerland). Membrane blocking and incubation with antibodies were done in a 5% w/v milk-TBST solution. Induced ABC transporters were labeled with mouse anti-FLAG® M2 (1:5000; Sigma-Aldrich). Rabbit anti-β-actin (1:5000; Sigma-Aldrich) antibody was used as loading control. Horseradish peroxidase coupled goat anti-mouse and goat anti-rabbit secondary antibodies (1:5000; Santa Cruz, Heidelberg, Germany) were used and detection done with the Super Signal West Pico Chemiluminescent Substrate according to the manufacturers protocol (Pierce, Socochim, Lausanne, Switzerland) by exposure to an X-ray film.

4.3.4 Immunocytochemistry

Cells from monoclonal lines were seeded onto 4-well Lab-Tek chamber slides (Nalge Nunc, Milian, Geneva, Switzerland) and induced with DOX (0.5 to 1 µg/mL in the culture medium) for 24 h for expression of ABC transporters and eGFP. As control, non-induced cells were used.

ABCB1-flag was analysed for plasma membrane localisation with monoclonal mouse anti ABCB1 antibody MRK 16 (Alexis Biochemicals, Lausen, Switzerland; dilution: 1:1000) directed against an extracellular epitope. Incubation with primary antibody was done in cell culture medium at 37°C for 1 h before fixation of the cells with 4% paraformaldehyde (PFA) in phosphate buffer (pH 7.4) for 10 min at room temperature (RT). Cy3 coupled goat anti-mouse secondary antibody (Jackson ImmunoResearch, Suffolk, UK; dilution: 1:500 in 5% w/v milk in PBS, 1 h, RT) was used for immunofluorescence detection.

ABCC1-flag membrane localisation was analysed by labeling ABCC1 after methanol fixation (30 s, -20°C) with monoclonal rat anti-ABCC1 antibody (Research Diagnostics Inc, Concord MA, USA. ; dilution: 1:100 in 5% w/v milk in PBS, 1 h, RT) recognizing an intracellular epitope, and co-labeling of the zona occludens protein ZO-1 with rabbit anti-ZO-1 (Zymed Laboratories, San Francisco CA, USA; dilution: 1:1000 in 5% w/v milk in PBS, 1 h, RT). For immunofluorescent detection Cy3-coupled goat anti-rat and Cy5-coupled goat anti-rabbit antibodies (Jackson ImmunoResearch, 1:500 in 5% w/v milk in PBS, 1 h, RT) were used.

Immunofluorescence was visualised by confocal laser scanning microscopy (Zeiss, LSM-510 Meta Jena, Germany) using a 100 x objective (numerical aperture 1.4) and sequential acquisition of separate channels. The pinhole was set to 1.0 Airy unit for each channel, and stacks of confocal sections (512 x 512) spaced by 0.3-0.5 µm were acquired at a magnification of 60-150 nm/pixel using the full dynamic range of the

photomultiplier. For display, images were processed with the image analysis software Imaris (Bitplane, Zurich, Switzerland). Images from both channels were overlaid (maximal intensity projection, "section" view) and background was subtracted when necessary.

4.3.5 Drug transport experiments

50000 (for MDCK-ABCB1 line) or 80000 (for MDCK-ABCC1 line) cells from an overnight culture of the respective clonal line were seeded on "Transwell" permeable supports (0.4 µm pore sise, polyester membrane, 6.5 mm diameter inserts in 24 well plate; Costar, Corning, New York, USA) and allowed to grow in culture medium for 3 days. Two days after seeding, 24 h prior to the transport assay, they were induced by addition of DOX (1 µg/mL for ABCB1 line and 0.5 µg/mL for ABCC1 line) to the medium if required. Transepithelial electrical resistance (TEER) was measured in Hanks Balanced Salt Solution (HBSS +Ca +Mg) (Gibco, Invitrogen) with an electrical volt-ohm meter (EVOM, World Precision Instruments (WPI), Berlin, Germany) and a corresponding Endohm-6 tissue resistance measurement chamber (WPI). Only "Transwells" with tightly grown cells that had a TEER ≥ 330 Ω*cm^2 were included in the analysis. When establishing the method, TEER was also measured after transport assay. Later on, TEER measurements at the end of the experiment were omitted to avoid loss of accuracy in amount of drugs found inside the cells. According to the polarisation of MDCK cells, the inside of a "Transwell" insert was defined as apical compartment (a) and the volume of the well below the insert was defined as basolateral compartment (bl) (*Figure 4.1 C*). The MDCK cell monolayer comprised the third, (intra-)cellular compartment (cells). On the third day after seeding the cells, drug transport experiments were performed. They were repeated at least 3 times with different culture batches in triplicate each. Substances (*see Table 4-1*) were added to the HBSS either into the apical (200 µL) (apical to basolateral transport (a → bl)) or the basolateral compartment (700 µL) (basolateral to apical transport (bl → a)). For every direction and condition (a → bl; bl → a; 4°C; 37°C), the "Transwells" were incubated for either 1 or 2 hours (Data for 2 h time-points not shown). Drug transport experiments were done in the presence of inhibitors (*see Table 4-1*) added to the apical and basolateral compartment to block endogenous ABC-transporters potentially expressed by MDCK cells. After the incubation, apical and basolateral solutions were collected separately and cells were treated with 200 µL 10 x trypsin (Gibco, Invitrogen), scratched from the membranes and 10, 11-

dihydrocarbamazepine (between 0.05 and 5 ng/mL) (dhCBZ; Sigma-Aldrich) was added to each sample as internal standard for mass spectrometry (MS) measurements. Samples were stored at -80°C before preparation for MS analysis.
In case of experiments with or without probenecid treatment the cell line MDCK-ABCB1 was used only in the non-induced state. The procedure was the same as above: 50'000 cells were seeded onto "Transwell" permeable supports and grown for 3 days. On day 2, the medium was changed but no DOX was added. After measuring the TEER, the medium was exchanged with HBSS containing either 0.5 mM probenecid (stock: 250 mM in dimethyl sulfoxide (DMSO), Sigma-Aldrich) or vehicle. Lamotrigine was added (*see Table 4-1*) only in the apical or the basolateral compartment depending on transport direction. No additional inhibitor was added. Incubation for both directions and conditions (with or without probenecid) were done at 37 °C and 4 °C for 1 hour.

Table 4-1: Substances and inhibitors used in transport assays:

	info	**concentration**	**used with**	**source**
Vinblastine	ABCB1 substrate	2 µM, stock (1000x) in water	MDCK-ABCB1 MDCK-ABCC1	vinblastine-sulfate, Sigma-Aldrich
Vincristine	ABCC1 substrate	2 µM, stock (500x) in water	MDCK-ABCC1	vincristine-sulfate, Sigma-Aldrich
Carbamazepine (CBZ)	AED	40 µM, stock (1000x) in 100 % ethanol	MDCK-ABCB1 MDCK-ABCC1	Sigma-Aldrich
Lamotrigine	AED	40 µM, stock (1000x) in 100 % ethanol	MDCK-ABCB1 MDCK-ABCC1	material transfer agreement, Desitin Pharma GmbH, Liestal, Switzerland
MK571	ABCC inhibitor	50 µM, stock (100x) in water	MDCK-ABCB1	Merck, Darmstadt, Germany
Tariquidar	ABCB inhibitor	0.2 µM, stock (100x) in 50 % acetonitrile	MDCK-ABCC1	tariquidar-mesilate; kind gift of Dr. D. Hermann, USZ, Zurich, Switzerland
Probenecid	organic anion transporter inhibitor	0.5 mM, stock (500x) in DMSO	MDCK-ABCB1 MDCK-ABCC1	Sigma-Aldrich

4.3.6 Sample preparation / extraction for MS

Cell-fractions were sonicated for 10 s prior to extraction. For CBZ and lamotrigine, the compounds (drugs and internal standard) were extracted using 1 volume of ethyl acetate (Chromasol V, Sigma-Aldrich) by vigorous vortexing for 30 s. The phases were separated by centrifugation at 6000 rpm for 6 min. The upper organic phase was dried at 65°C and samples were reconstituted in 50% acetonitrile (Chromasol V, Sigma-Aldrich) containing 0.1% formic acid (Sigma-Aldrich) and filtered (Cronus regenerated cellulose membrane filters, diameter 4 mm, pore size 0.45 µm, Infochroma AG, Zug, Switzerland) prior to LC-MS/MS analysis.

For the more hydrophilic vinblastine and vincristine, no organic extraction could be carried out. Instead, samples were directly supplemented with 1 volume of acetonitrile and 0.1% formic acid and filtered prior to MS analysis.

4.3.7 LC - MS / MS analysis

The separation of vinblastine and the internal standard dhCBZ was performed on an Agilent 1100 liquid chromatography system using an eclipse XDB-C18 reverse phase column (Agilent, 4.6 x 150 mm, 5 µm pore size) with a corresponding opti-guard pre-column. The mobile phase consisted of (A) 0.1% aqueous formic acid (Sigma-Aldrich) and (B) acetonitrile containing 0.1% formic acid at a flow rate of 500 µL/min using an injection volume of 20 µL. Starting conditions of 50% buffer B were maintained for 2 min, followed by a linear gradient from 50 to 90% B within 6 min. An isocratic flow of 90% B was held for 1 min and finally the column was re-equilibrated for 1.5 min with 50% B. The HPLC system was coupled to a 4000 QTRAP hybrid quadrupole linear ion trap mass spectrometer (Applied Biosystems, Carlsbad CA, USA) equipped with a TurboV source and electrospray (ESI) interface. Analytes were recorded using multiple reaction monitoring (MRM) in positive mode (+MRM) using the following source specific parameters: IS 5'500 V, TEM 400°C, curtain gas (CUR = 10), nebuliser gas (GS1 = 40), heater gas (GS2 = 50) and collision gas (CAD = 5). The compound specific parameters (listed in *Table 4-2*) for the different substrates were determined by direct infusion of standard solutions (100-300 nmol/mL) in acetonitrile at a flow rate of 10 µL/min using the quantitative optimisation function of the Analyst software 1.4.2 (Applied Biosystems).

Table 4-2: Parameters used for MS analysis and quantification of substances:

Compound	Transition	DP	CE	CXP	EP	LOD (ng/mL)	LOQ (ng/mL)
Vinblastine	811.5 → 224.2	91	59	18	10	0.7	2.4
Vincristine	413.3 → 362.1	71	27	10	10	0.03	0.1
dhCBZ	239.0 → 194.2	26	31	14	10	0.0003	0.001
CBZ	237.0 → 194.1	26	27	14	10	0.06	0.18
Lamotrigine	256.04 → 211.0	31	56	14	10	5.2	17.4

DP (declustering potential), CE (collision energy), CXP (collision cell exit potential), EP (entrance potential), LOD (limit of detection), LOQ (limit of quantification)

Vincristine recording was also done in +MRM mode with the following source specific parameters: IS 5'500 V, TEM 450 °C, curtain gas (CUR = 10), nebuliser gas (GS1 = 40), heater gas (GS2 = 50) and collision gas (CAD = 5). Starting conditions of 50 % buffer B at a flow rate of 500 µL/min were maintained for 2 min, followed by a linear gradient from 50 to 90 % B within 6 min. An isocratic flow of 100 % B was held for 1 min and finally the column was re-equilibrated for 1.5 min with 50 % B. The injection volume was 20 µL.

CBZ and lamotrigine were also recorded using +MRM with the following source specific parameters, listed in the order CBZ- before lamotrigine-settings, if differing: IS 5'500 V, TEM 450 °C respectively 400 °C, curtain gas (CUR = 10), nebuliser gas (GS1 = 15 or 40 respectively), heater gas (GS2 = 40) and collision gas (CAD = 5). Starting conditions of 20 % or 50 % buffer B, respectively, at a flow rate of 400 µL/min or 500 µL/min were maintained for 2 min, followed by a linear gradient from 20 to 100 % B, respectively 50 to 90 % B within 3 min. An isocratic flow of 100 % B, respectively 90 % B was held for 3 min and finally the column was re-equilibrated for 3 min with 20 % B or 50 % B respectively. The injection volume was 10 µL for CBZ and 20 µL for lamotrigine.

4.3.8 Assay validation example

Samples were quantified by determining the area under the curve (AUC) with the quantification function of the Analyst® software 1.4.2 (Applied Biosystems), using the transitions listed in Table 4-2 for each substrate. The background noise was assessed by analyzing a blank area of the chromatogram obtained with the standard curve

which was generated using blank samples spiked with a series of concentrations ranging from 0.01-10'000 ng/mL depending on the substance. Hence, correlation coefficients as well as the limit of detection (LOD) and the limit of quantification (LOQ) could be obtained. The LOD was corresponding to a signal-to-noise ratio of 3 and the LOQ to a signal to noise ratio of 10, respectively.

4.3.9 Data analysis

The integrals of the transported substance were compared to the corresponding standard curve (ranging from 0.01 to 10'000 ng/mL depending on the substance). Best fit was obtained by using a double logarithmic scale with concentrations on the x and counts of the AUC on the y axis. The concentrations were calculated using the regression curves of the standard curves ($x = (y/a)^{(1/b)}$, where $y = \int AUC$, a it the axis intercept of the slope at 1 ng/mL, b is the slope) and then multiplied with the dilution factor (D) which resulted from the preparation. The concentration of dhCBZ, the internal standard (IS), was used to normalise the measurement and exclude preparation and pipetting errors. As the absolute values varied between experiments the calculated yield of IS (Y_{IS}) and the concentrations of the substances were in percentages ($C_{eff} = (x * D)/Y_{IS}) * 100$). These were compared between the three compartments to calculate the percentage of total recovered substance for each compartment as part of 100 %.

Each transport experiment was performed in triplicate. These were compared and outliers determined using Grubbs outlier test for 3 samples (r_m (95%, 3) = 1.153). The resulting mean was considered as one experiment. For each condition, at least three experiments were performed.

Statistical analyses to compare means across conditions were done using one-tailed Mann-Whitney test (Graph Pad, Prism 4). A *P* value ≤ 0.05 was considered as statistically significant.

Transport ratios (TRs) for ABCB1, an exporter located on the apical side of cells, were calculated as follows: TR = (% substance $apical_{bl \rightarrow a}$)/(% substance basolateral $_{a \rightarrow bl}$). TRs for ABCC1, an exporter located on the basolateral side of cells, were calculated as follows: TR = (% substance basolateral $_{a \rightarrow bl}$)/(% substance $apical_{bl \rightarrow a}$). The corrected TR (cTR) was calculated by dividing the TR obtained from induced cells by the TR obtained from non-induced cells. A substance was considered to be a substrate of the corresponding transporter, if cTR ≥ 1.5.

4.4 Results

4.4.1 Immunochemical detection of induced transporters

Cell extracts were prepared from induced and non-induced MDCK cells after 24 h exposure to DOX or vehicle, respectively. Western blotting using anti-flag antibodies revealed strong expression of ABCB1-flag in the MDCK-ABCB1 cell line and ABCC1-flag in the MDCK-ABCC1 cell line. No signal was detected in non-induced (vehicle-treated) MDCK-ABCB1 or MDCK-ABCC1 cells or in non-transfected cells, indicating absence of transcriptional activation in the absence of DOX, as well as specificity of antibodies (*Figure 4.2 A).*

In preliminary experiments, DOX concentrations were titrated to achieve maximal surface expression of the respective ABC transporter in each cell line as assessed by ICC. A concentration of 1 µg/mL cell culture medium was found optimal for ABCB1- and 0.5 µg/mL for ABCC1 induction (data not shown). Higher DOX concentrations resulted in decreased cell surface expression and increased intracellular accumulation, possibly due to overload of the protein sorting machinery. Immunofluorescence with an antibody targeted to an extracellular epitope of ABCB1 was used to assess its distribution in induced cells. The expected polarised localisation of ABCB1 in the apical membrane compared to cytoplasmic GFP was evident when examining stacks of confocal images along the z-axis (*Figure 4.2 B*). In case of MDCK-ABCC1 cells, the basolateral localisation of ABCC1 immunofluorescence (using an antibody against an intracellular epitope) was verified by comparing its localisation to that of the tight-junction marker ZO-1 (*Figure 4.2 C*). The specificity of immunofluorescence staining was verified in non-induced cells, in which only background staining was observed. Examination of both monoclonal cell lines in the microscope revealed, unexpectedly, a mosaic-like expression pattern of the recombinant proteins. Therefore, despite transcription being driven from a single promoter and the cells being of monoclonal origin, it was not possible to achieve a homogeneous expression or to use eGFP fluorescence as an index of recombinant transporter expression on a single-cell basis.

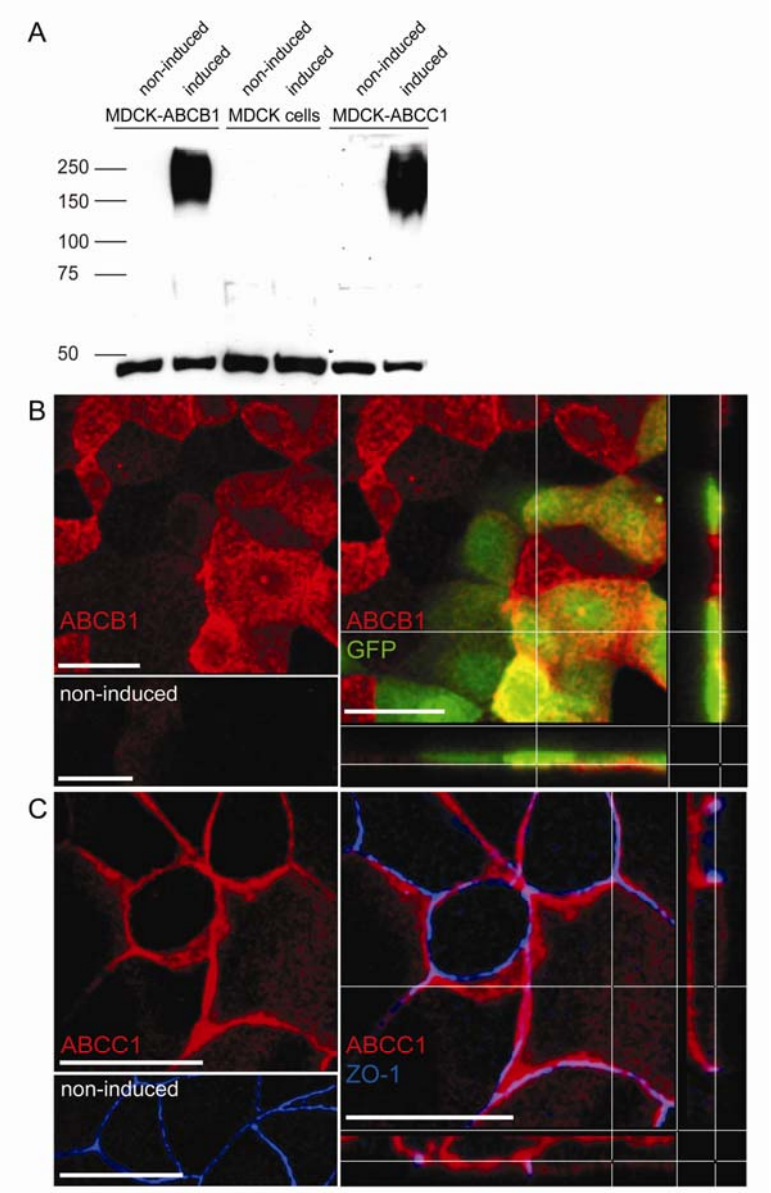

Figure 4.2: Verification of induced expression and correct subcellular localisation of ABC transporters.

A: *Immunoblot analysis of cell extracts with anti-flag antibodies showing two prominent bands around 200 kDa in induced MDCK-ABCB1 and MDCK-ABCC1 cells. No flag-immunopositiv band was found in the non-transfected MDCK cells (middle lanes). The bands at 42 kDa represent β-actin as loading control.* ***B*** *and* ***C:*** *Images from confocal laser scanning microscopy in top and side views (z-stacks) showing the expression and membrane localisation of transporters in transfected MDCK cells upon induction of gene expression. No signal is evident in and non-induced cells (low left hand corner).* ***B:*** *ABCB1 (red) localises apically above the cytoplasmic eGFP (green) expressed in a co-inducible manner. Scale bars: 30 µm* ***C:*** *ABCC1 localises on the basolateral side of cells below the tight junction marked by the protein ZO-1 (blue). Scale bars: 20 µm*

4.4.2 Design of drug transport experiments

For transport experiments with ABCB1 and ABCC1, one monoclonal cell line was selected for each of them, based on optimal expression levels and subcellular targeting, as shown above. Cells were grown on a filter insert in 24-well plates to form a monolayer dividing the well into two compartments: apical (a) and basolateral (bl) in addition to the intracellular compartment (*Figure 4.1* C). The basolateral part of

the cells was in contact with the filter. Each experiment was done in triplicate and in parallel at 37º and at 4ºC with cells pre-exposed to DOX (induced) or to vehicle (non-induced), yielding 12 samples per assay. All drugs were assayed in both directions (a → bl and bl → a) for 1 h and 2 h. Qualitatively similar results were obtained with both incubation times, but the variability between experiments was larger after 2 h (data not shown). The TEER was stable over 1 h but tended to decrease after 2 h. All results presented here are derived from the 1 h incubation time.

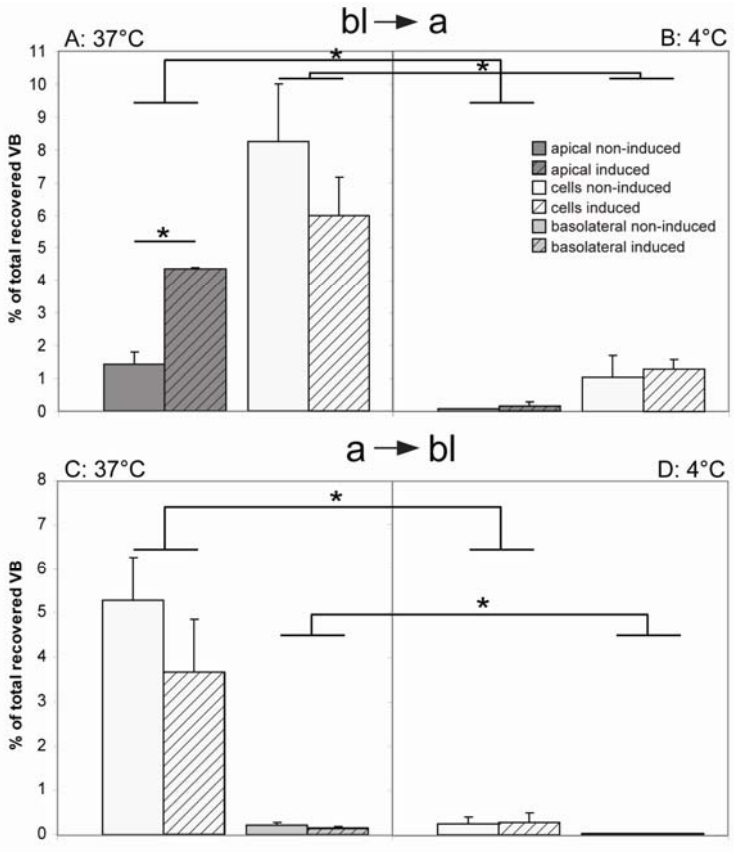

Figure 4.3: Verification of functionality of MDCK-ABCB1 cells with vinblastine (VB) as transport substrate. *Data represent means of percentages of total recovered vinblastine from the apical, cellular and basolateral compartment of the "Transwell". Error bars represent the standard deviation (SD) obtained from at least 3 experiments, each in triplicate. For clarity only the percentages of drug recovered from the compartments where it was not added are displayed. Histograms show fractions from apical compartment (dark grey), cells (white) and basolateral compartment (light grey) from left to right. Representations of fractions from induced conditions are striped, non-induced ones are plain.* ***A:*** *Transport from bl → a for 1h at 37°C. There is a significant difference between the fractions recovered from apical compartments of induced compared to non-induced cells (*P ≤ 0.05, Mann-Whitney).* ***B:*** *Transport from bl → a for 1h at 4°C. C and D: Transport from a → bl at 37°C and 4°C, respectively. There are significant differences for all compartments between the transport at 37°C and at 4°C (*P ≤ 0.05, Mann-Whitney). (*P ≤ 0.05, Mann-Whitney).*

At the end of each experiment, the samples from the three compartments (apical, basolateral, and intracellular) were collected and their drug content analysed by LC-MS/MS. The concentration of dhCBZ added as internal standard to determine the LOD and LOQ was adjusted for each compound separately as their detection sensitivities were different (*Table 4-2*).

4.4.3 Vinblastine transport in ABCB1-MDCK cells

To test the functionality of induced ABCB1 expression, we measured transport of vinblastine, a known substrate for ABCB1 (Shalinsky et al., 1993), using 0.05 ng/mL dhCBZ as internal standard and MK571 as ABCC inhibitor (*Table 4-1*). To rule out any bias by endogenous expression of ABCC proteins, MK571 was added in all experiments to both sides of the system. As expected from the apical location of ABCB1, vinblastine was transported more efficiently in the bl → a direction by cells overexpressing ABCB1 (induced) than by control cells (non-induced). The percentage of total vinblastine recovered from the apical compartment after 1 h incubation was three times higher than that obtained from non-induced cells (4.4% versus 1.4%, $P \leq 0.05$, n = 6, Mann-Whitney, one-tailed) (*Figure 4.3 A*), representing a cTR of 4.73 at 37°C (*Table 4-3*). Strikingly, the intracellular fraction of vinblastine was larger than the apical fraction in bl → a directed transport, indicating good penetration of the drug across the basolateral membrane and intracellular accumulation. However, functionality of ABCB1 was also supported by the observation that vinblastine added to the apical compartment tended not to enter cells as efficiently in induced cells compared to non-induced cells (*Figure 4.3 C*, $P = 0.1$). Furthermore, little vinblastine reached the basolateral compartment (~ 0.2%) when added to the apical side, independently of induction of ABCB1 expression and efficient substrate uptake (4 – 5% intracellular fraction). This result indicates that only negligible, if any diffusion of vinblastine occurred between the monolayer formed by MDCK cells.

When assayed at 4°C, vinblastine transport was significantly reduced compared to physiological temperature for both induced and non-induced cells (*Figure 4.3*). A minute fraction of substrate was transported under these conditions (0.07 – 0.15% in bl → a direction) but the contribution of ABCB1-overexpression cells was still evident (cTR = 2.49). Additionally, compared to 37°C, we found a significantly reduced intracellular fraction of vinblastine, suggesting energy-dependent intracellular accumulation. Altogether, these findings demonstrate specific, directional, and active transport of vinblastine in induced ABCB1-MDCK cells.

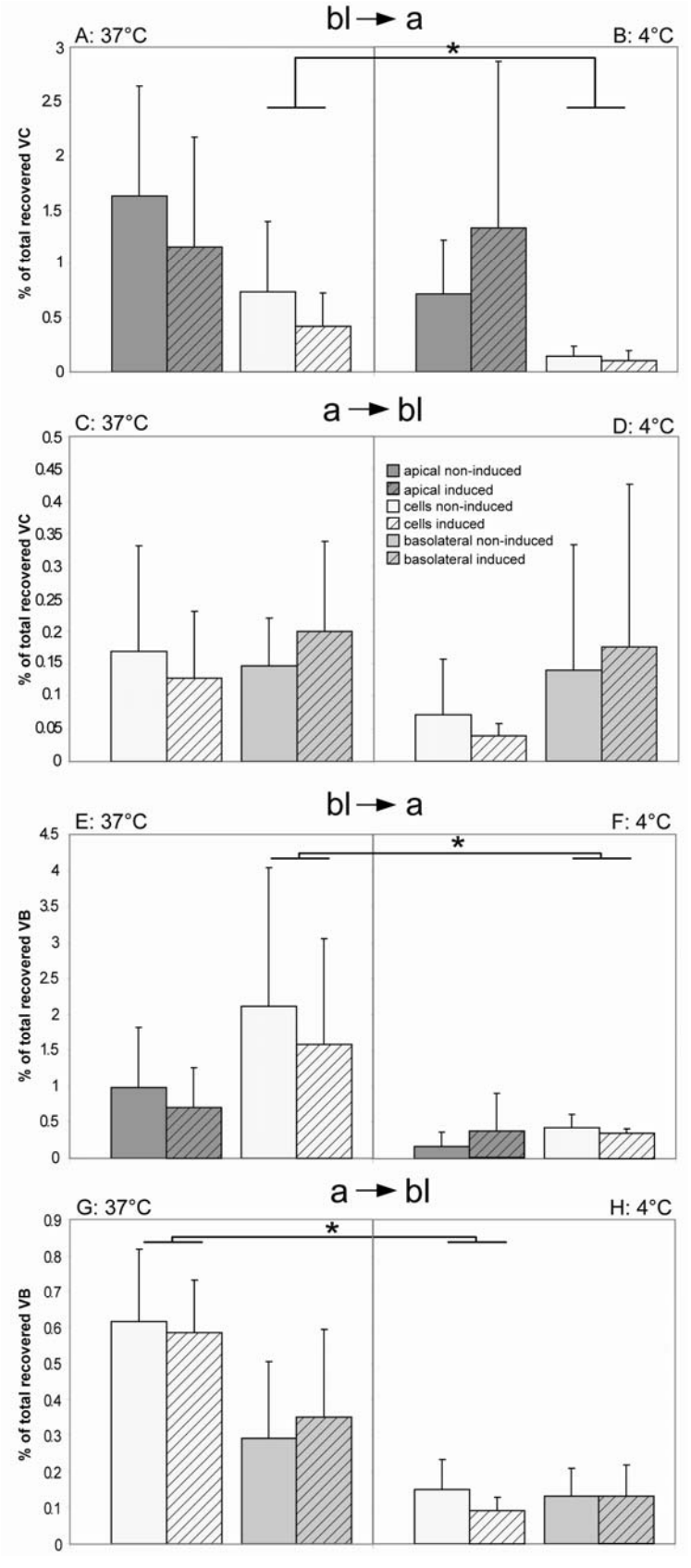

Figure 4.4: Analysis of functionality of transport by MDCK-ABCC1 cells. *Means of fractions of total recovered drug (± SD) for each compartment are displayed.* ***A*** *and* ***B:*** *Transport of vincristine (VC) from bl → a at 37°C and 4°C, respectively. The fraction of transported drug is very small compared to Figure 4.3. No significant differences were found between the induced and non-induced cells (P = 0.1, Mann-Whitney). Significantly less vincristine is transported at 4°C compared to 37°C (*P = 0.05, Mann-Whitney).* ***C*** *and* ***D:*** *Transport of vincristine (VC) from a → bl at 37°C and 4°C respectively. Almost nothing penetrates the cells. No significant differences were detected.* ***E*** *and* ***F:*** *Transport of vinblastine (VB) from bl → a at 37°C and 4°C, respectively. The only significant difference was detected between the cellular fractions at 37°C and 4°C (*P = 0.05, Mann-Whitney).* ***G*** *and* ***H:*** *Transport of vinblastine (VB) from a → bl at 37°C and 4°C, respectively. No significant differences were detected except between the cellular fractions at 37°C and 4°C (*P = 0.05, Mann-Whitney).*

4.4.4 Vincristine transport in ABCC1-MDCK cells

The functionality of MDCK-ABCC1 cells was assessed with its substrate vincristine (Mao et al., 2000) and with vinblastine (*Figure 4.4*), using 0.05 ng/mL dhCBZ as internal standard for both compounds. Addition of tariquidar in all experiments ensured to rule out potential transport by endogenous ABCB1. Unexpectedly, the monoclonal cell line selected was found to be highly impermeable for these two compounds. Only a minute fraction of vincristine penetrated the cells, in particular from the apical side (<1%). Hence, we could not detect significant differences in a → bl directed transport between the various conditions tested (*Figure 4.4 C, D*). However, in the bl → a direction, we detected slightly less transport in the presence of ABCC1 and obtained a cTR of 1.92 for vincristine at 37°C (*Table 4-4*), arguing for ABCC1-mediated extrusion. However, due to the small amount of drug transported, the differences between induced and non-induced cells were not significant (2.5% and 1.5%, respectively, $P = 0.1$, one-tailed Mann-Whitney; *Figure 4.4 A*). At 4°C, the fraction of drug transported was even smaller than at 37°C (*Figure 4.4 B, D*).

The low permeability of this monoclonal cell line was confirmed with vinblastine transport assays, especially in the a → bl direction (*Figure 4.4 G, H*) compared to ABCB1-MDCK cells. Similar to vincristine, the cTR at 37°C (cTR = 1.69) was suggestive of ABCC1-mediated transport, although the differences in drug amounts recovered in the three compartments were statistically non-significant when compared to non-induced cells (*Figure 4.4 E-H*).

4.4.5 Analysis of carbamazepine and lamotrigine as substrates for ABCB1

The results so far indicate that inducible expression of ABCB1 allows measuring directional drug transport across a monolayer of MDCK cells. Therefore, we tested whether the two AEDs CBZ and lamotrigine are potential substrates of ABCB1 in this system. Both drugs were assayed at a concentration comparable to plasma-levels in longitudinally treated patients. Thus, they were 20 times more concentrated than vincristine or vinblastine. Transport assays were performed with MK571, an inhibitor of ABC-transporters of the ABCC subfamily (*Table 4-1*). The internal standard dhCBZ was used in a concentration of 5 ng/mL with the CBZ samples and 1 ng/mL with the lamotrigine samples.

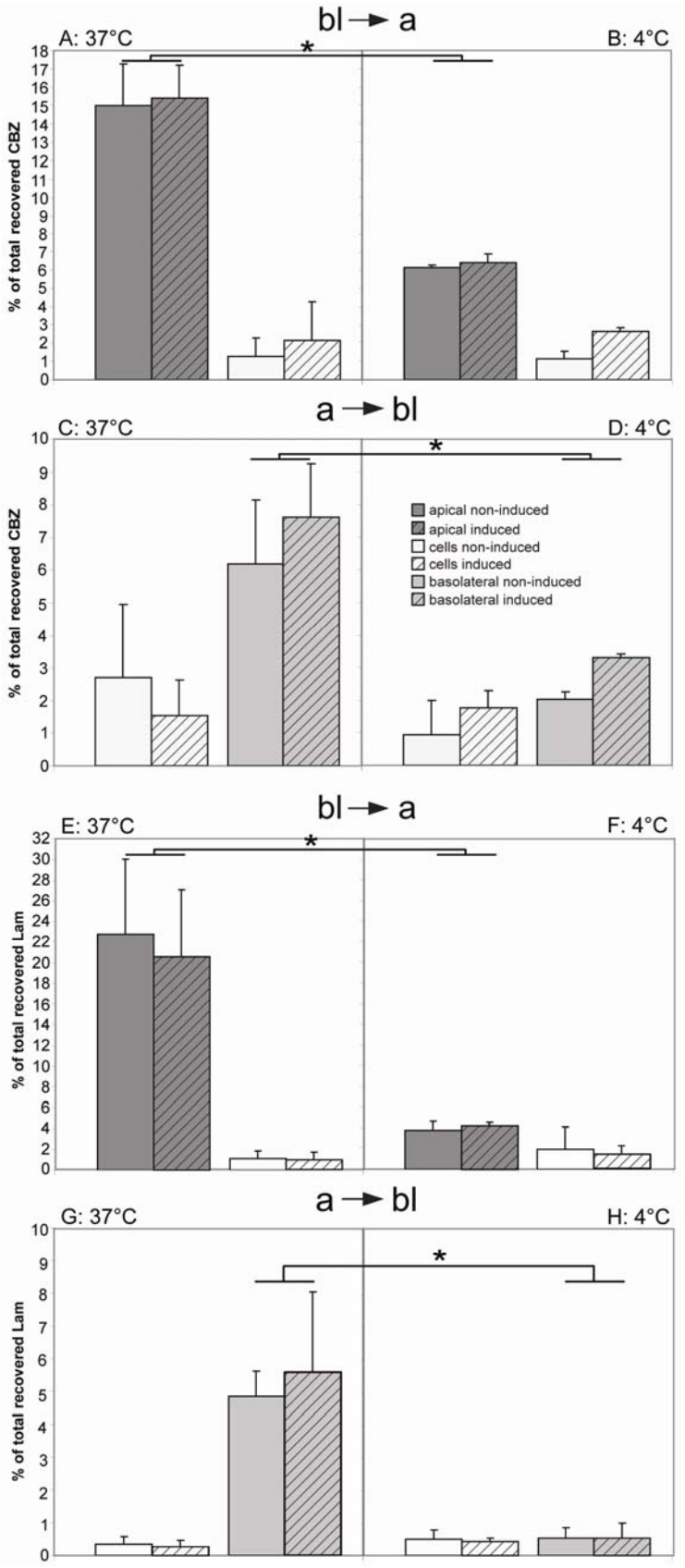

Figure 4.5: Analysis of CBZ (A to D) and lamotrigine (Lam) (E to H) transport by MDCK-ABCB1 cells. *Data represent means (± SD) of percentages of total recovered drug as described for Figure 4.3* ***A*** *and* ***B:*** *Transport of CBZ from bl → a at 37°C and 4°C, respectively.* ***C*** *and* ***D:*** *Transport of CBZ from a → bl at 37°C and 4°C, respectively. No significant differences were detected except between 37°C and 4°C for the apical and basolateral compartments (*P ≤ 0.05, Mann-Whitney).* ***E*** *and* ***F:*** *Transport of lamotrigine from bl → a at 37°C and 4°C, respectively.* ***G*** *and* ***H:*** *Transport of lamotrigine from a → bl at 37°C and 4°C, respectively. No significant differences were detected except between 37°C and 4°C for the apical and basolateral compartments (*P ≤ 0.05, Mann-Whitney).*

CBZ: No selective CBZ transport by ABCB1 could be detected as CBZ accumulation in apical, cellular and basolateral compartments did not differ significantly between ABCB1-expressing (induced) and control (non-induced) cells as shown in Figure 4.5 A-D.

Accordingly, the cTR calculated for CBZ was around 1 (*Table 4-3*). However, a large fraction of CBZ was transported across the cells, independently of ABCB1 induction. CBZ transport was temperature-sensitive, evident by roughly 2.5 – 3 fold reduction of bl → a and a → bl transport at 4°C compared to 37°C ($P \leq 0.05$, one-tailed Mann Whitney) (*Figure 4.5 B, D*). Transport was directional, with about twice as much CBZ being transported from bl → a (~ 15% of total recovered CBZ in the apical compartment) than in the a → bl direction (~ 7% recovered in the basolateral compartment) (*Figure 4.5 A, C*). This difference also holds true at 4°C (~ 6% found apically$_{bl \rightarrow a}$; ~ 2 - 3% found basolaterally$_{a \rightarrow bl}$; *Figure 4.5 B, D*). A substantial passive transport of CBZ remained evident at 4°C, as expected for a lipophilic drug. Additionally, it is noticeable that the intracellular fraction of CBZ (~ 1 – 2% of total CBZ independently of transport direction and temperature), represents a high concentration, which would be available for transport by ABCB1. Altogether, these results suggest that endogenous transporters contribute to bidirectional CBZ translocation across MDCK cell monolayers, whereas overexpression of ABCB1 could not be shown to influence it.

Lamotrigine: ABCB1 likewise could not be shown to influence lamotrigine transport. As shown in Figure 4.5 E-H, there was no significant difference between induced and non-induced cells and the cTR was close to 1 (*Table 4-3*). Similar to CBZ, lamotrigine transport was found to be strongest in the bl → a direction. About 20% of total lamotrigine was recovered in the apical compartment after bl → a directed transport, compared to about 5% in the opposite direction (*Figure 4.5 E, G*). Moreover, the cells were even more permeable to lamotrigine than to CBZ when assayed at 37°C. Lamotrigine transport was also temperature-sensitive independently of induction of ABCB1 expression. The transport rate was reduced between 5 fold (bl → a) and 10 fold (a → bl) ($P \leq 0.05$, one-tailed Mann-Whitney) at 4°C compared to 37°C. The fraction of lamotrigine transported at 4°C in the bl → a direction (~ 4%) and in the a → bl direction (~ 0.5%) (*Figure 4.5 F, H*) was smaller than that measured for CBZ (*Figure 4.5 G and H*), indicating that lamotrigine does not readily cross the cells by passive diffusion, despite its limited solubility in water.

Table 4-3: Transport ratios and corrected transport ratio for transport in ABCB1-MDCK cells (in bl → a direction for an apically expressed exporter).

	Vinblastine		**Carbamazepine**		**Lamotrigine**	
	37°C	4°C	37°C	4°C	37°C	4°C
TR induced	30.88	6.55	1.81	1.94	3.68	7.75
TR non-induced	6.52	2.63	2.57	3.04	4.71	6.95
cTR	**4.73**	**2.49**	**0.71**	**0.64**	**0.78**	**1.12**

(TR = apical$_{bl \rightarrow a}$ / basolateral$_{a \rightarrow bl}$; cTR = TR$_{ind}$ / TR$_{non\text{-}ind}$)

4.4.6 Analysis of carbamazepine and lamotrigine as substrates for ABCC1

Despite the very low permeability of the ABCC1-MDCK cell clone used for vincristine and vinblastine, transport of CBZ and lamotrigine, which are less hydrophilic, was assessed in these cells as well. Tariquidar was added during the transport as ABCB inhibitor and the concentrations of dhCBZ as internal standard were again 5 ng/mL for CBZ and 1 ng/mL for lamotrigine measurements. The amounts of AED were adjusted to plasma levels from patients and were 20 x higher than concentrations of vinblastine or vincristine.

CBZ: The fraction of CBZ actively transported at 37°C in either direction (*Figure 4.6 A and C*) across the MDCK-ABCC1 cell monolayer was much larger than that of vincristine and vinblastine. ABCC1-MDCK cells even transported more CBZ than MDCK-ABCB1 cells, independently of induction. These differences indicate that CBZ readily penetrates into MDCK cells and that endogenous transport mechanisms can vary among clonal cell lines, underscoring the importance of using an inducible system to assess drug transport with an appropriate control. Additionally, we again observed that MDCK cells are generally more permeable for CBZ in bl → a direction than in a → bl. This difference was also seen at 4°C.

As for ABCB1, CBZ could not be demonstrated to be transported by ABCC1 in induced cells (*Figure 4.6 A-D*). The cTR was around 1 (*Table 4-4*). Failure to detect ABCC1-mediated transport occurred despite the substantial amount of CBZ accumulated intracellularly (1%). Again, a pronounced difference between CBZ transport at 37°C

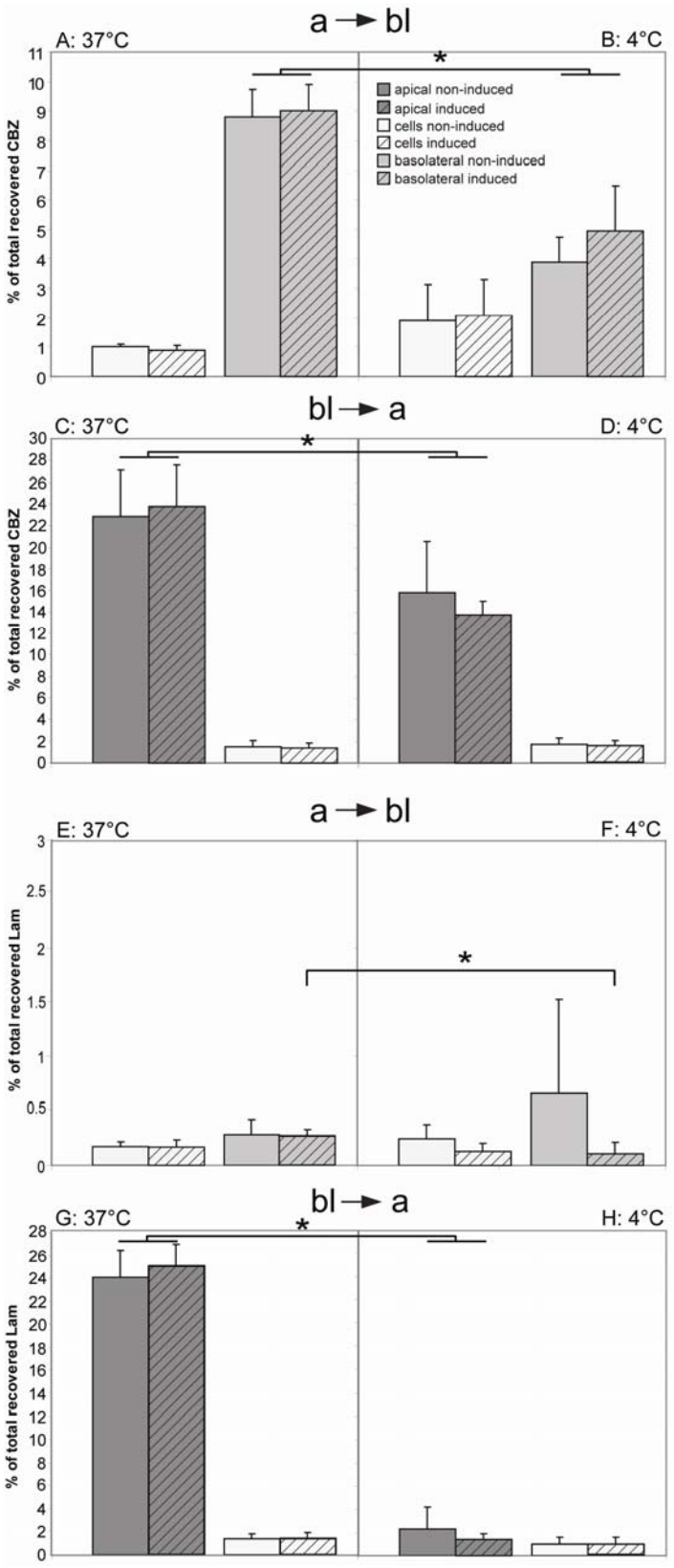

Figure 4.6: Analysis of CBZ (A-D) and lamotrigine (Lam) (E-H) transport by MDCK-ABCC1 cells. *Representations of means (± SD) of fractions of recovered drug as described for Figure 4.3.* ***A*** *and* ***B:*** *Transport of CBZ from a → bl at 37°C and 4°C, respectively.* ***C*** *and* ***D:*** *Transport of CBZ from bl → a at 37°C and 4°C, respectively. No significant differences were detected except between 37°C and 4°C for the apical and basolateral compartments (*P ≤ 0.05, Mann-Whitney).* ***E*** *and* ***F:*** *Transport of lamotrigine from a → bl compartment at 37°C and 4°C, respectively.* ***G*** *and* ***H:*** *Transport of lamotrigine from bl → a at 37°C and 4°C, respectively. No significant differences were detected except between 37°C and 4°C for the basolateral and apical compartments in the induced case of apical to basolateral directed transport and for apical and basolateral compartments in both cases, in the basolateral to apical direction (*P ≤ 0.05, Mann-Whitney).*

Table 4-4: Transport ratios and corrected transport ratio for transport in MDCK-ABCC1 cells (in a → bl direction for a basolaterally expressed exporter). Values below 1 indicate transport in the opposite direction, here in bl → a.

	Vincristine		**Vinblastine**		**Carbamazepine**		**Lamotrigine**	
	37°C	4°C	37°C	4°C	37°C	4°C	37°C	4°C
TR induced	0.17	0.31	0.50	0.34	0.38	0.36	0.01	0.08
TR non-induced	0.09	0.20	0.29	0.76	0.39	0.25	0.01	0.28
cTR	**1.92**	**0.67**	**1.69**	**0.45**	**0.98**	**1.47**	**0.92**	**0.27**

(TR = basolaterala→bl / apicalbl→a; cTR = TRind / TRnon-ind)

and at 4°C was observed independent of induction of ABCC1-expression, pointing to endogenous active transport mechanisms in the MDCK-ABCC1 cell line. The residual transport at 4°C (~ 15% total recovered CBZ apically$_{bl\rightarrow a}$; ~ 4% basolaterally$_{a\rightarrow bl}$; *Figure 4.6 B and D*) was reduced 2.3-fold for a → bl and 1.5-fold for bl → a as compared to 37°C.

Lamotrigine: The fraction of lamotrigine recovered apically (~25%) following bl → a directed transport was very high, whereas only minute amounts of lamotrigine reached the basolateral side (~ 0.3%) when added to the apical compartment (*Figure 4.6 E and G*). These results show that the MDCK-ABCC1 cell line formed a tight barrier and had a strong directional preference for lamotrigine transport. The 12-fold reduction in bl → a transport at 4°C (~ 2% lamotrigine found apically$_{bl\rightarrow a}$; ~ 0.1 – 0.6% found basolaterally$_{a\rightarrow bl}$) (*Figure 4.6 F and H*) revealed that lamotrigine was mainly transported in an energy-dependent manner. The small fractions found inside the cells (~ 1.5% for bl → a; ~ 0.2% for a → bl) compared to what was found on the receiving side also hint to powerful active extrusion mechanisms.

Despite considerable intracellular accumulation of lamotrigine, comparison of induced and non-induced cells yielded a cTR around 1 (*Table 4-4*) and no significant differences between total recovered drug in the induced and the non-induced state were detected (*Figure 4.6*).

4.4.7 Analysis of endogenous transport mechanisms

As we observed that CBZ and even more so lamotrigine are efficiently transported across MDCK cells at 37°C independently of ABCB1 and ABCC1 by energy-dependent

mechanisms, we attempted to block this endogenous transport with probenecid, a broad-band inhibitor of organic anion transporters.

These experiments were conducted for lamotrigine using non-induced cells derived from the ABCB1 monoclonal line. In this assay no additional inhibitors were used. In the absence of probenecid, we observed bi-directional transport of lamotrigine at 37°C, which was largely abolished in experiments performed at 4°C and was stronger in the bl → a direction, as observed in control cells (see *Figure 4.5*). Addition of probenecid (0.5 mM) to both compartments during lamotrigine transport had no statistically significant effect on the relative lamotrigine concentration recovered in the various compartments, although the variability between experiments was rather high for no discernable technical reason (*Figure 4.7*).

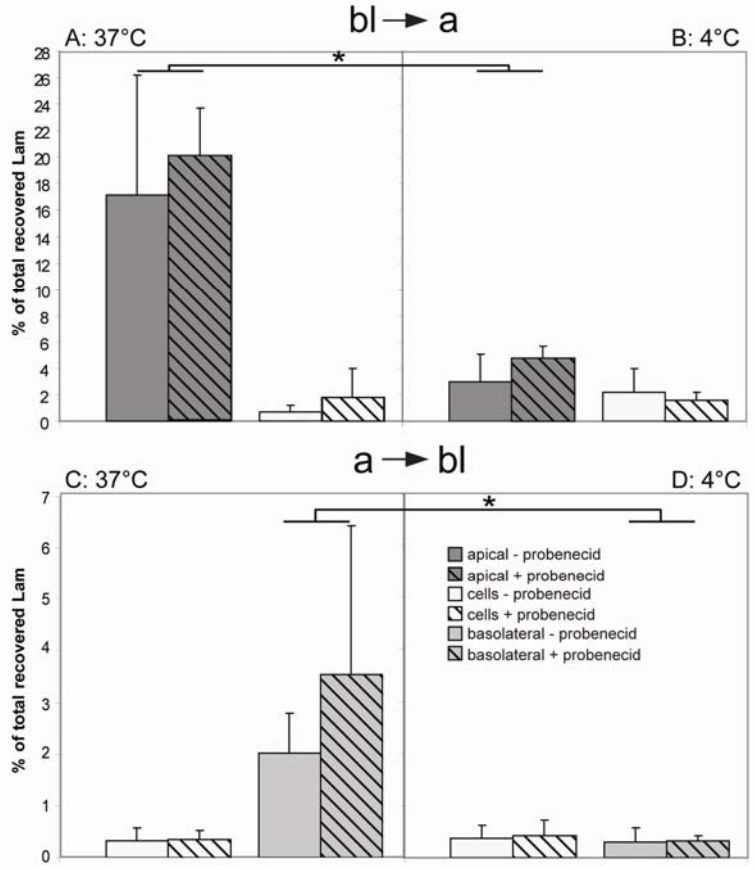

Figure 4.7*: Analysis of endogenous lamotrigine (Lam) transport**. *To inhibit endogenously expressed organic anion transporters, probenecid was added during the transport experiments (+ probenecid) and compared to vehicle treated controls (- probenecid). For each case 4 trials were performed on different days, each in triplicate. Data represent means (± SD) of percentages of total recovered drug for each compartment (apical, cells, basolateral).* ***A *and* ***B:*** *Transport of lamotrigine from the bl → a at 37°C and 4°C, respectively.* ***C*** *and* ***D:*** *Transport of lamotrigine from a → bl at 37°C and 4°C respectively. No significant differences were detected except between 37°C and 4°C for the apical and basolateral compartments (P ≤ 0.05, Mann-Whitney).*

4.5 Discussion

We established an *in vitro* model which is well suited to study transport of drugs by either human ABCB1 or ABCC1 through a biological barrier. Upon induction with DOX, the multidrug transporters are expressed and trafficked to their appropriate position. By testing the ABCB1 transport system with a known substrate, and at 37°C and at 4°C, we could show that our in vitro BBB model is functional and specific. Induction of ABCB1 led to a significant increase in vinblastine extrusion, resulting in a cTR of 4.7. Control experiments of vincristine and vinblastine transport by ABCC1-MDCK cells were not satisfactory due to insufficient penetration of these drugs unlike that seen in ABCB1 cells. We found no evidence for ABCB1- or ABCC1-mediated transport of CBZ or lamotrigine, suggesting that these AED may not be substrates of ABCB1 or ABCC1. However, we observed in both cell lines a prominent active transport of CBZ and lamotrigine independently of ABCB1 or ABCC1 expression, which might have obscured a contribution of the induced transporters. Our results confirm previous findings for CBZ (Owen et al., 2001; Baltes et al., 2006) but contrast with a recent report of lamotrigine transport by ABCB1 (Luna-Tortos et al., 2008).

4.5.1 Characterisation of basal transport in non-induced MDCK cells

A major advantage of an inducible expression system is that the same cell clone used to study transport serves as a baseline control for experiments conducted in parallel under identical conditions. Importantly, recombinant ABCB1 and ABCC1 were not detectable in non-induced cells, confirming that the promoter used here was silent in the absence of DOX. Furthermore, the sensitivity of LC-MS/MS provided reliable results even for the intracellular compartment or for conditions under which little transport occurred, such as 4ºC. A further prerequisite for measuring drug extrusion is the presence of a tight barrier preventing passive drug diffusion between cells. MDCK cells proved highly suitable for this purpose, since they appeared largely impermeable for the four substrates tested, but exhibited distinct, energy-dependent bidirectional transport capacity independently of ABCB1 and ABCC1 induction.

Nevertheless, the control experiments with vincristine in the ABCC1 system revealed the limitations of this approach. The MDCK-ABCC1 cell line was found to be virtually impermeable for vincristine and vinblastine. This result indicates that these substrates did not penetrate intracellularly, and therefore could not be assayed reliably. The

reasons why vinblastine was transported into MDCK-ABCB1, but not into MDCK-ABCC1 cells, is unknown. However, this finding shows that different clones of MDCK cells cannot readily be compared with each other, stressing the need for inducible expression to have an adequate control. Despite the limited penetration of vincristine into MDCK-ABCC1 cells, the cTR pointed towards active extrusion from induced cells.

Both cell lines exhibited a clear directional preference for transport of CBZ and lamotrigine at physiological temperatures, with more substrate crossing the cells in the bl → a direction than vice-versa. The large fraction of lamotrigine transported, notably by the MDCK-ABCC1 cell line, suggests at first glance that the monolayer might not be impermeable. However, only a small fraction (<0.3%) was translocated in the opposite direction, ruling out this concern. In the a → bl directed transport, the fraction of lamotrigine recovered intracellularly (~ 0.2 – 0.3%) was lower than in the opposite direction (~ 1 – 1.5%) for both the MDCK-ABCB1 and the MDCK-ABCC1 lines. From these observations, we conclude that directional transport is ensured by endogenous mechanisms facilitating basolateral drug penetration and apical extrusion.

Passive drug diffusion across the monolayer contributes to most drug transport under conditions where energy-dependent pumps are inhibited, such as lowering the temperature to 4°C. This might also affect membrane permeability by changing its physico-chemical properties and lateral mobility of its constituent lipids and proteins, thereby contributing to reduced drug transport at 4°C. The high fraction of CBZ translocated in these control experiments suggested that CBZ easily crosses lipid bilayers, despite being more water-soluble than lamotrigine. Interestingly, we still observed a directional transport preference for both drugs at 4°C. This effect likely reflects the larger area of the basolateral membrane compared to the apical membrane, offering more surface for passive diffusion (von Bonsdorff et al., 1985). Another possibility is the different molecular composition of the apical and basolateral membranes (Richardson and Simmons, 1979, van Meer and Simons, 1982) which might favor passive diffusion through the basolateral membrane.

The prominent endogenous transport capacity of both cell lines for CBZ and lamotrigine prompted us to test whether organic anion transporters, such as OAT1, which is highly abundant at the basolateral membrane of kidney tubular cells (Burckhardt and Burckhardt, 2003) mediate this effect. Addition of probenecid, a broad-band inhibitor of OATs, to the lamotrigine transport assays resulted in variable effects, with a blockade of basolateral to apical transport being evident in 2/4 tests.

The reasons for this variability could not be determined. It should be noted, however, that probenecid has been reported to enhance ABCC2 activity (Huisman et al., 2002, Zimmermann et al., 2008). Considering the fact that MDCK cells display a range of endogenous drug carriers including multidrug transporters like ABCC2 (Goh et al., 2002), it is conceivable that the inhibition of OATs by probenecid might have been masked by activation of ABCC2 and possible transport mediated by organic anion transporter polypeptides (OATPs) which are, among many other transporters, also present in MDCK cells (Ito et al., 2005)

4.5.2 Transport capacity of induced cells

Analysis of transporter expression upon DOX induction by Western blotting and immunocytochemistry revealed a strong expression and correct subcellular localisation of recombinant ABCB1 and ABCC1 (*Figure 4.2*). When tested with vinblastine, a known substrate for ABCB1, the functionality of recombinant ABCB1 could be demonstrated. The cTR obtained in this system (4.73 at 37°C) was slightly higher than that reported in previous studies (Baltes et al., 2007, Luna-Tortos et al., 2008). It was striking that a large fraction of drug (~ 6 – 8%; bl → a) could accumulate intracellularly in the presence of ABCB1; this result suggests that the endogenous transporter(s) responsible for vinblastine uptake are more efficient than ABCB1-mediated extrusion. Despite this positive control, overexpression of either ABCB1 or ABCC1 had no influence on CBZ or lamotrigine transport, irrespective of the direction, duration of the assay, or temperature (*Figure 4.5 &Figure 4.6; Table 4-3 &Table 4-4*). These negative results argue against ABCB1- and ABCC1-mediated CBZ and lamotrigine transport under the present experimental conditions. However, we cannot rule out that the strong endogenous transport present in MDCK cells might mask transport by ABCB1 or ABCC1. Especially since a recent study found weak, tariquidar-sensitive, directional lamotrigine transport by ABCB1 in a concentration equilibrium assay after prolonged transport duration (Luna-Tortos et al., 2008). We found directional lamotrigine transport as well, but independent of ABCB1 induction.

In brain, ABCB1 is localised on the apical, luminal side of microvessels. Our findings indicate that ABCB1 might not be involved in epilepsy-related pharmacoresistance to CBZ or lamotrigine; it cannot be excluded that it may still play a role in pharmacoresistance to other AEDs, or that its function in endothelial cells is regulated differently than in MDCK cells. In case of ABCC1, its basolateral targeting results in an opposite distribution in endothelial cells (brain side) and choroid plexus epithelial cells

(blood side). Therefore, ABCC1-mediated drug extrusion might occur at the blood-cerebrospinal fluid barrier. In line with this possibility, ABCC1 may even play a role in delivering drugs to the brain, as shown after ischemia (Kilic et al., 2008). Although our results do not demonstrate its participation in CBZ and lamotrigine transport; it remains to be analysed whether ABCC1 might play a role in refractoriness to AED treatment.

4.5.3 Conclusions

To sum up, we have established an inducible *in vitro* model of the BBB which is suitable to study the substrate specificities of recombinant ABC-transporters with high sensitivity. With this system we found no evidence that ABCB1 or ABCC1 are involved in the transport of CBZ and lamotrigine. We can not exclude that other, so far undetermined transporters present at the BBB, might play a central role in drug-refractory epilepsy. Possible candidates include other multidrug transporters of the ABC superfamily, which have been found to play a role in the limited access of the drug topotecan to the brain (de Vries et al., 2007). Alternatively, drug extrusion mediated by ABCB1 and ABCC1 from endothelial cells might be dependent on mechanisms that are absent in MDCK cells.

4.6 Acknowledgements:

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4.7 Authors' contributions

Baldinger, M.H.: Design of study, participation in molecular cloning of vectors, establishment of stably transfected cell lines including biochemical and ICC analyses, conduction of transport experiment including sample preparation for MS analysis, evaluation of MS data, statistical analyses, literature research, manuscript preparation. **Cronin, A.:** Programming of LC-MS/MS device to measure and quantify amounts of compounds in the samples, critical reading of manuscript, advisory input. **Tilen, R.:** Execution and analysis of one set of transport studies as part of a diploma thesis. **Schwerdel, C.:** Technical support for molecular biology and cell cultures. **Arand, M.:** Provision of LC-MS/MS device, critical reading of manuscript, advisory input. **Fritschy, J.-M.:** Head of research group, grant applications, design of the study, advisory input, surveillance of project, editing of manuscript. **Krestel, H.E.:** Design of the study, literature research, grant application, cloning of expression vectors, data analysis, editing of manuscript, experimental and advisory input.

4.8 References:

See Reference section page 98.

5 Results: *in vivo* study

Role of the multidrug transporter Abcb1a in pharmacoresistance to carbamazepine in a mouse model of temporal lobe epilepsy

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[a]These authors contributed equally to this work.

Submitted

5.1 Abstract

Purpose: It has been hypothesised that pharmacoresistance to antiepileptic drugs (AEDs), which occurs frequently in temporal lobe epilepsy (TLE), underlies insufficient bioavailability of the drugs at the epileptic focus. This could be explained by export of drugs at blood-brain barrier (BBB) level. In this regard the focus of many studies was laid on an involvement of multidrug transporters of the ATP-binding cassette (ABC) family which are expressed at the BBB. Understanding a possible interaction of AEDs with multidrug exporters is crucial for future development of better treatments. Here, we aimed to investigate the role of the ABC transporter Abcb1a on carbamazepine (CBZ) resistance in a mouse model of TLE.

Methods: The effect of CBZ on healthy animals was analysed by studying its sedative action. Hence, general activity and locomotion was assessed in wild type (WT) and transporter-deficient mice. To study the involvement of Abcb1a in pharmacoresistance, WT and abcb1a$^{0/0}$ mice were rendered epileptic by intrahippocampal kainate (KA) injection and the effect of CBZ on seizure frequency was analysed.

Results: Treatment with 5 or 10 mg/kg CBZ produced strong sedation in all genotypes. In epileptic mice, effect of CBZ on seizure frequencies varied between genotypes, but differences could not clearly be assigned to the lack or presence of Abcb1a.

Discussion: Genotype-specific differences hint to an involvement of Abcb1a in resistance to AED. However, this could not be unambiguously demonstrated, pointing to an involvement of additional mechanisms.

Key words: temporal lobe epilepsy, intrahippocampal kainic acid injection, mouse model, antiepileptic drug resistance, ABC transporter

5.2 Introduction

Temporal lobe epilepsy (TLE) with hippocampal sclerosis (HS) is a common type of focal epilepsy and often associated with pharmacoresistance. The mechanisms underlying medically intractable seizures are elusive, since patients typically do not respond to a broad range of antiepileptic drugs (AEDs) with various modes of action. Two major hypotheses have been proposed: i) Changes in drug targets which may be pre-existent or derive from neuropathological alterations in the epileptic brain (Jandova et al., 2006). ii) Impaired penetration of AEDs into the brain, caused for instance by drug-metabolism in the blood-brain barrier (BBB) or by overexpression of functional multidrug exporters (Loscher, 2002, Sisodiya et al., 2002). Evidence supporting an increased expression of multidrug transporters of the ATP binding cassette (ABC) superfamily come from analyses of brain tissue from patients with drug-resistant epilepsy (e.g. (Dombrowski et al., 2001, Sisodiya et al., 2002) and experimental studies showing seizure-induced expression of specific ABC transporters in animal models of TLE (Zhang et al., 1999, Rizzi et al., 2002, Sills et al., 2002, Volk et al., 2004).

Among the best characterised ABC transporters, the multidrug resistance protein ABCB1 (MDR1 or P-gp) is highly abundant in blood-tissue barriers such as the BBB and in organs with secretory functions where its main physiological role is to extrude xenotoxic compounds. Rodents have two isoforms, Abcb1a and Abcb1b, encoded by distinct genes, with complementary tissue distribution. Mainly Abcb1a is present at the BBB (Schinkel et al., 1994), localised on the luminal (apical) membrane of brain microvessel endothelial cells (Soontornmalai et al., 2006, Roberts et al., 2008).

ABCB1 extrudes a wide range of structurally and functionally diverse compounds and can render cancer cells resistant to certain drugs when overexpressed; hence it might play a role in resistance to AEDs (Kwan and Brodie, 2005). However, data about transport of AEDs is controversial. Carbamazepine (CBZ), a first line AED for the treatment of partial complex seizures, was not found to be an ABCB1 substrate using several *in vitro* approaches, or *in vivo* using healthy abcb1a/1b$^{0/0}$ mice (Owen et al., 2001a, Baltes et al., 2006, Luna-Tortos et al., 2008, Rivers et al., 2008) Nevertheless, in another study using microdialysis in rat brain, higher levels of CBZ were found if Abcb1 was blocked by verapamil infusion (Potschka et al., 2001). Additionally, a study conducted in abcb1a$^{0/0}$ mice reported higher brain/serum concentration ratios for CBZ and other AEDs compared to wild type (WT) despite comparable serum levels (Sills et

al., 2002). Irrespective of these controversies, other ABC transporters possibly involved in AED resistance include ABCC1 (Mrp1) and ABCC2 (Mrp2) (Abcc1 and Abcc2 in rodents) of the ABCC subfamily (Loscher and Potschka, 2002), as well as breast cancer resistance protein (BCRP or ABCG2) (Aronica et al., 2005, van Vliet et al., 2005).

Here, we aimed to clarify the role of multidrug transporters in CBZ resistance. The TLE model based on a unilateral intrahippocampal injection of KA in adult mice has the advantage to produce a focal lesion and induce frequent recurrent seizures (typically 30 - 100/hour) (Arabadzisz et al., 2005). This model is well suitable to study the effect of AEDs on TLE. We have shown previously that CBZ, as well as valproate and phenytoin, but not diazepam, failed to suppress recurrent spontaneous seizures in this model (Riban et al., 2002, Gouder et al., 2004), providing an excellent opportunity to replicate such experiments in mutant mice.

To evaluate the role of multidrug transporters in restricting brain access of CBZ during physiological and pathophysiological conditions, we analysed here the sedative action of CBZ in healthy animals and its anti-convulsive action in the intrahippocampal KA mouse model of TLE. Analysis of general motor activity and locomotion following systemic CBZ administration was taken as surrogate for sedation (Trevor and Way, 1995). Mutant mice lacking the abcb1 or abcc1 genes generated in the FVB strain were compared to WT FBV mice and to C57BL6/J mice. We have shown previously that Abcc2, while normally expressed in liver and kidney, is not detectable immunohistochemically in the BBB of FVB mice (Soontornmalai et al., 2006), suggesting a potential absence of Abcc2 in the BBB. The involvement of Abcb1a in resistance to the antiepileptic action of CBZ was analysed in KA-treated mice displaying chronic recurrent seizures. Hence, seizure frequency and duration were measured by electroencephalographic (EEG) recordings before and after treatment with CBZ or vehicle in abcb1a$^{0/0}$, FVB, and NMRI mice. The latter served as baseline for comparison with previous studies (Riban et al., 2002, Gouder et al., 2004), whereas FVB mice provided a measure of the potential role of Abcc2 in influencing the action of CBZ.

5.3 Materials and methods

5.3.1 Animals

Adult mice were purchased from commercial suppliers: Swiss NMRI (Harlan, Horst, The Netherlands) served as WT control in the TLE model. C57BL/6J (Jackson Laboratories, Bar Harbor, USA) were used as WT control in behavioural experiments as they are an inbred strain and mice are similar in size to FVB mice (Taconic Farms, Lille Skensved, Denmark). FVB is the WT background strain of the transporter-deficient abcb1a$^{0/0}$ and abcc1$^{0/0}$ mice (Taconic Farms). Mouse lines were maintained at the Institute of Pharmacology and Toxicology, University of Zurich. Mice were housed in a 12 h light-dark cycle with free access to food and water. All procedures were approved by the local veterinary authorities and were performed in accordance with the European Community Council Directive (86/609/EEC). A maximum of 36 animals per genotype was used for this study.

5.3.2 Measurement of general motor activity and locomotion

Measurement of motor activity in rodents represents a standard behavioural assay for testing the sedative potential of drugs (Trevor and Way, 1995). General motor activity (including exploration and grooming) and locomotion were measured in four genotypes (n = 16 per genotype, all male), C75BL/6, FVB, abcb1a$^{0/0}$ and abcc1$^{0/0}$ mice to assess the sedative action of CBZ. Mice were housed individually in circular arenas for 1 week prior to the start of the experiments to habituate and were kept there all the time. Activity recordings were taken over three hours in the individual automated circular arenas equipped with four equidistant photocells (Imetronic, Pessac, France). Locomotion was scored by counting the number of interruptions of two consecutive photocells, whereas general motor activity was scored when only one photocell was interrupted. CBZ in 2-hydroxypropyl-β-cyclodextrin complex (HBC) (RBI, Natick, MA, U.S.A.) was dissolved in distilled water at a final concentration of 2.5 mg/mL (0.5 mL/kg). Mice received an i.p. injection of either vehicle or CBZ (5 mg/kg and 10 mg/kg) and were placed back into their individual circular arena. Each animal served as its own control by comparing its behaviour following vehicle injection to that after either lower dose or higher dose of CBZ with an interval of two days between injections. Statistical analysis of paired treated groups was performed using

two-way repeated measure analysis of variance (ANOVA) for comparison between the four genotypes (GraphPad, Prism 4). Post-hoc comparisons versus control conditions (vehicle) were performed using the Bonferroni test (GraphPad, Prism 4, La Jolla, CA, USA). Statistical significance was set at $P \leq 0.05$.

5.3.3 Surgery and electrode implantations

A new group of female mice of the NMRI, FVB and abcb1a$^{0/0}$ genotypes was used for treatment with KA. Mice were anaesthetised with 2.5 – 3% Isofluorane (Baxter, Volketswil, Switzerland) and each mouse was stereotactically injected into the right dorsal hippocampus (anteroposterior (AP) = −1.8 mm, mediolateral (ML) = −1.6 mm, dorsoventral (DV)= −2.0 mm, with bregma as reference) with 140 nL of a 5 mM solution of kainic acid (KA) (Tocris, Lucerna Chem AG, Lucerne, Switzerland) in saline (0.9% NaCl, i.e., 1 nmol). For EEG recordings, mice were implanted under anaesthesia with bipolar electrodes aimed at the right dorsal hippocampus, using the same coordinates (AP = −1.8 mm, ML = −1.6 mm, DV = −2.0 mm), either immediately after KA injection or two weeks later, as described (Riban et al., 2002). Following surgery, the mice were allowed to recover from anaesthesia, injected with 1mg/kg buprenorphine (Temgesic, Essex Chemicals, Lucerne, Switzerland) and then returned to their home cages with daily monitoring of wellbeing.

5.3.4 Electroencephalographic recordings and drug administration

EEGs were recorded in freely moving KA-treated mice using a digital acquisition system (AcqKnowledge MP100, Biopac Systems Inc., Santa Barbara, CA, USA; sampling rate, 200 Hz). Recordings were performed following habituation of the mice to the test cage, around midday to minimise potential circadian variations. To assess the effects of AEDs on the frequency and duration of recurrent seizures 8 - 14 mice per genotype were recorded at three weeks post KA treatment. EEG recordings were analysed off-line and artefacts due to animal movements were discarded. Paroxysmal events were defined as seizures when they lasted more than 10 s and were separated from each other by intervals of at least 3 s. For each animal, the average number of seizures per minute and seizure duration was calculated. Only those with more than six epileptic paroxysmal discharges per hour and appropriate anatomical lesion in the hippocampus (n = 8 - 10, per genotype) were included in the study. To assess the effect of ABC transporters on drug resistance, pure CBZ (Sigma-Aldrich, Buchs,

Switzerland) was dissolved in 60% PEG400 (Sigma-Aldrich) in distilled water at a final concentration of 10 mg/mL. This formulation allowed to administer higher doses than in previous studies (Riban et al., 2002, Gouder et al., 2004). A dose of either 20 mg/kg or 40 mg/kg of CBZ or vehicle only was injected i.p. in randomised order during the EEG recording session. Mice were left to recover for at least two days between two sessions. Prior to drug treatment, baseline EEG of seizure activity was recorded for 180 min, and following drug administration the animals were recorded for a further 180 min. Each animal was thus used as its own baseline control. For some mice not all drug-concentrations could be tested due to loss of the implanted electrodes. A final number of four to seven mice per genotype and condition could be evaluated.

5.3.5 Statistical analysis of drug effects

The effects of CBZ were assessed by comparing the frequency and duration of hippocampal paroxysmal discharges recorded during the first 100 min period post-drug administration, with seizures recorded prior to drug injection. Counting was started 15 min after drug administration to rule out effects due to the stress of injection and handling. Due to large inter-individual differences in seizure frequency, the baseline was normalised to 100% for quantification. Statistical analysis to identify effects of treatment or genotype was done by two-way ANOVA followed by a post-hoc Bonferroni test to compare the effect of drug to vehicle (GraphPad, Prism 4). Analysis of effect to baseline was done by two-tailed Wilcoxon signed rank test (GraphPad, Prism 4). Significance level was set at $P \leq 0.05$.

5.3.6 Histological controls

After completion of the experiments (4 - 5 weeks after KA injection), mice were deeply anaesthetised with pentobarbital (Nembutal, 40 mg/kg i.p.) and killed by decapitation. The brains were rapidly excised and frozen with powdered dry ice. Parasagittal sections were cut at 14 µm with a cryostat, mounted onto gelatine-coated slides and dried at room temperature for approximately 12 h. Histological analysis was performed after Nissl (cresyl violet) staining to verify the location of the bipolar electrode and the morphological alterations caused by KA injection.

5.4 Results

5.4.1 Strain-dependent sedative effects of carbamazepine

Treatment with CBZ can cause adverse side-effect at the therapeutic dose, including sedation, ataxia, dizziness, and visual disturbances (Albani et al., 1995).To evaluate the potential role of Abcb1, Abcc1 and Abcc2 in limiting the action of CBZ in the brain, we measured its sedative effect in C57BL/6J, FVB, abcb1a$^{0/0}$ and abcc1$^{0/0}$ mice during the light-phase using an automated device to track general motor activity and locomotion (*Figure 5.1*). Data analysis was performed in the time-window between 15 and 45 min after CBZ administration (*Figure 5.1 B*) to avoid inclusion of the stress-induced increase in activity caused by the injection (*Figure 5.1 A*).

CBZ administration caused a marked decrease in general activity and locomotion during the entire 45 min period analysed. Already at a dose of 5 mg/kg of CBZ, two-way ANOVA revealed a significant genotype effect on general activity ($F_{3,28}$ = 11.99, P < 0.0001; n=8 per genotype) and on locomotion ($F_{3,28}$ = 4.664, P = 0.0091) as well as a significant treatment effect on general activity ($F_{1,28}$ = 44.67, P < 0.0001) and on locomotion ($F_{1,28}$ = 20.92, P < 0.0001). This was evidenced by reduction of both general activity as well as locomotion (*Figure 5.1 B*) in FVB, abcb1a$^{0/0}$ and abcc1$^{0/0}$ mice (post-hoc Bonferroni, P < 0.05), but not in C57BL/6J mice.

At a dose of 10 mg/kg, CBZ-induced sedation was more pronounced (two-way ANOVA, drug effect on general activity: $F_{1,28}$ = 132.4, P < 0.0001; drug effect on locomotion: $F_{1,28}$ = 67.04, P < 0.0001) and no genotype effect was observed, since all four strains were affected to the same extent for the locomotor activity (two-way ANOVA, not significant (ns)), whereas slight differences in general activity were seen between genotypes ($F_{3,28}$ = 4.317, P = 0.0127) (*Figure 5.1 B*).

These results show that WT and mutant mice derived from the FVB strain are more sensitive to moderate concentrations of CBZ than C57BL/6J mice, even though activity levels after vehicle treatment were similar across all genotypes. Neither Abcb1a nor Abcc1 appear to reduce the sedative action of CBZ in this test, whereas the higher sensitivity of FVB mice compared to C57BL6/J mice to a low dose of CBZ might be due to the absence of Abcc2 at the BBB.

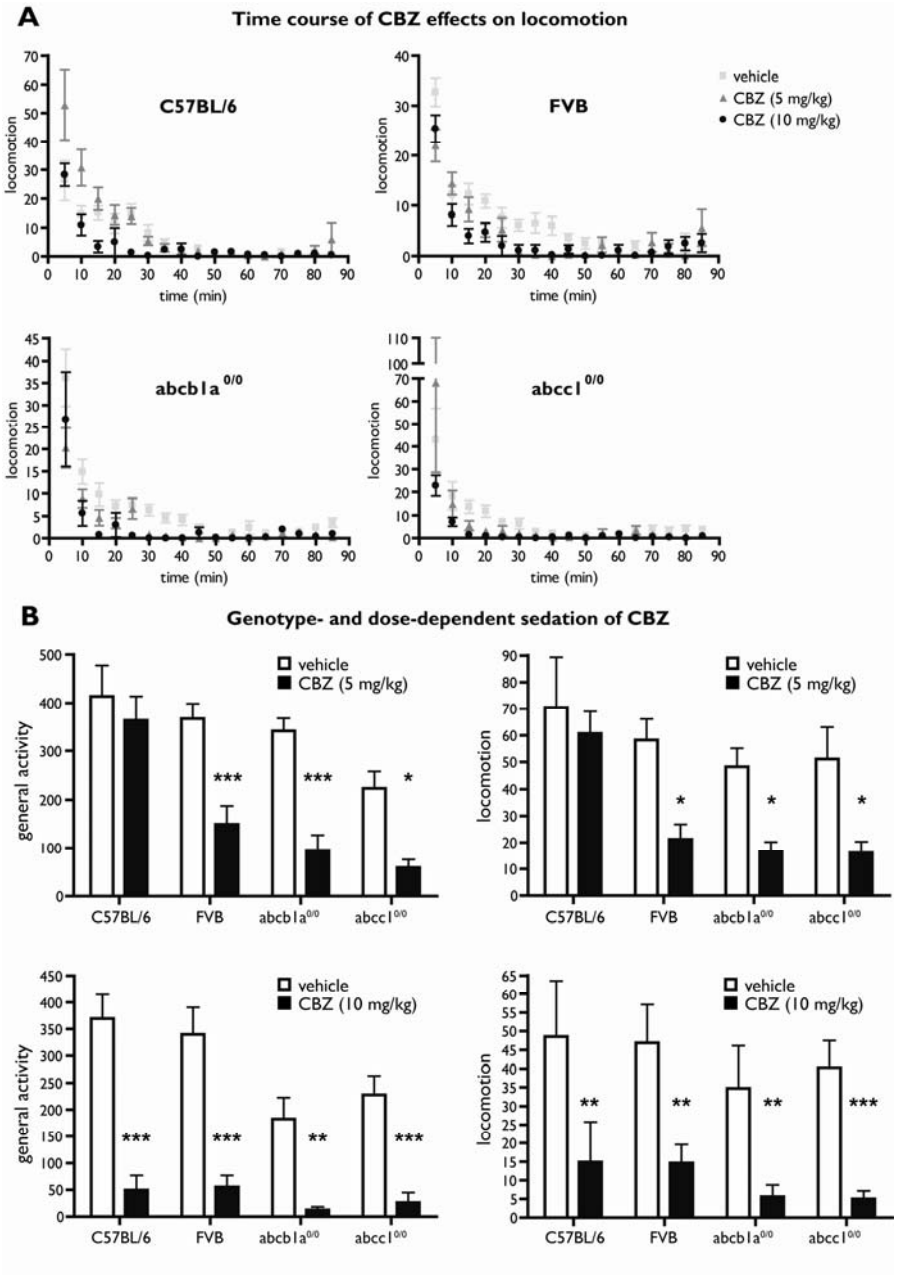

Figure 5.1: Effect of CBZ on locomotion and general motor activity in C57BL/6J, FVB, abcb1a$^{0/0}$ and abcc1$^{0/0}$ mice. A: *Locomotion (± standard error of the mean (SEM)) was plotted against time. The sedative effect of CBZ (5 mg/kg and 10 mg/kg) compared to vehicle injection is seen as decreased locomotion over time for each genotype. Sedation was most prominent within the first 45 min and started to subside after about 60 min. Following vehicle injection mice became hyperactive and calmed down after about 45 min. Injection-related stress was observed in all conditions during the first 15 min.* ***B:*** *Effect of CBZ on general activity and locomotion in C57BL/6J, FVB, abcb1a$^{0/0}$ and abcc1$^{0/0}$ mice. Both were scored 15 - 45 min following injection. Top panels: Following CBZ (5 mg/kg) injection, general motor activity and locomotion were not altered in C57BL/6J mice but was significantly reduced in FVB, abcb1a$^{0/0}$ and abcc1$^{0/0}$ mice. Bottom panels: After receiving a higher dose of CBZ (10 mg/kg), mice from all genotypes showed significantly reduced general and locomotor activity. All results are presented as mean ± SEM, n = 8 per genotype. Statistics: post-hoc Bonferroni test. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.*

5.4.2 Development of chronic epilepsy following intra-hippocampal kainic acid injection

These experiments were performed in KA-treated NMRI, FVB and abcb1a$^{0/0}$ mice during the phase of chronic recurrent seizures (see below). The doses of CBZ tested were higher than for assessing motor activity (20 mg/kg and 40 mg/kg). Under these conditions, all mice, independently of strain, were strongly sedated for up to 2 - 3 h after CBZ injection, confirming the results above.

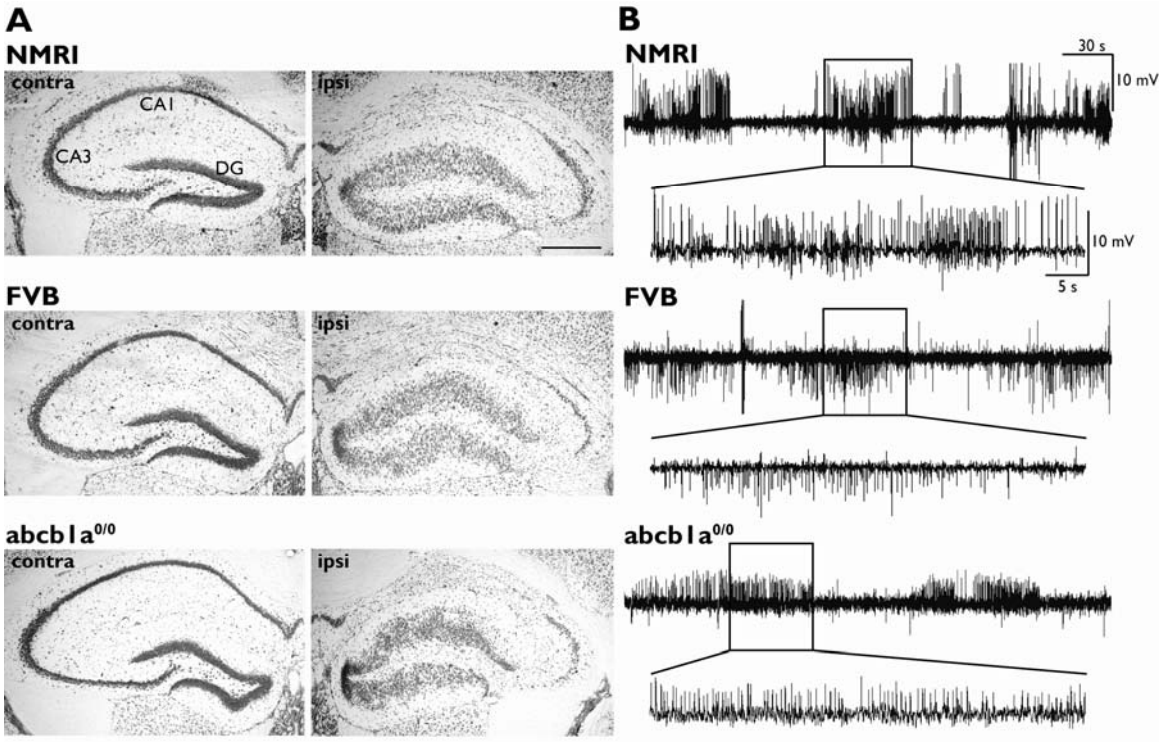

Figure 5.2: Effects of intrahippocampal KA injection in NMRI, FVB, abcb1a$^{0/0}$ and abcc1$^{0/0}$ mice. A: *The morphological changes occurring 4 - 5 weeks post KA injection are shown in Nissl-stained sections; panels on the left side represent the non-injected, contralateral hippocampus (contra) and are compared to the injected, ipsilateral hippocampus (ipsi) represented on right side. Extensive cell loss in the KA-injected hippocampus can be seen in CA1, CA3 and the hilus of the dentate gyrus (DG), together with a marked dispersion of the dentate granule cells. Scale bar: 500 µm* ***B:*** *Examples of representative EEG recordings of spontaneous recurrent seizures in chronically epileptic mice. Paroxysmal rhythmic discharges typical of epileptic seizures are best seen at high temporal resolution. Seizure frequency and durations varied between individuals but no significant differences between genotypes could be determined (see Table 5-1).*

As a consequence of KA injection, three distinct stages of seizure progression can be seen both morphologically and electrophysiologically in the mouse hippocampus (Bouilleret et al., 1999, Bouilleret et al., 2000, Riban et al., 2002). To analyse whether these changes occurred to a similar extent in the strains analysed here and to verify the placement of electrodes, histological analysis was performed at the conclusion of the experiments, 4 – 5 weeks after KA injection (*Figure 5.2 A*), corresponding to the chronic phase (Bouilleret et al., 1999). Examination of Nissl-stained sections revealed no difference in hippocampus cytoarchitecture between genotypes; in all mice, extensive neuronal loss was evident in the hilus, CA1 and CA3 areas together with the prominent dispersion of the dentate granule cell layer characteristic of this model. These alterations were restricted to the dorsal hippocampus and no modifications were observed contralaterally, as reported previously (Bouilleret et al., 1999, Knuesel et al., 2001, Riban et al., 2002, Arabadzisz et al., 2005). EEG recordings performed during the chronic phase revealed that KA injection led to spontaneous recurrent seizures in all genotypes. Typically, the seizures started with high voltage sharp waves at low frequency followed by higher frequency lower voltage rhythmic spike-and-wave activities characteristic of paroxysmal epileptic discharges (*Figure 5.2 B*). The frequency of seizures per minute and the duration of each seizure were comparable between genotypes but varied inter-individually and intra-individually between different days of recording. Strikingly, the inter-individual variability in FVB and abcb1a$^{0/0}$ mice (6 – 8 fold differences) was higher than in NMRI mice (~ 3 fold differences). The length of seizures was similar for all genotypes (*Table 5-1*). Spontaneous seizures were observed during all experimental recording sessions, until the mice were sacrificed for histological analysis.

Table 5-1: Seizure durations before and after treatment with vehicle or CBZ in the different genotypes

genotype	baseline	vehicle	CBZ (20 mg/kg)	CBZ (40 mg/kg)
NMRI	35.3 ± 14.1	25.5 ± 2.6	34.6 ± 9.2	40.9 ± 38.0
FVB	24.6 ± 6.8	18.5 ± 4.3	21.7 ± 5.6	19.9 ± 4.6
abcb1a$^{0/0}$	25.5 ± 6.4	25.1 ± 7.3	28.8 ± 11.8	18.8 ± 3.7

Results (in sec) are given as mean ± SD, n = 4 – 7

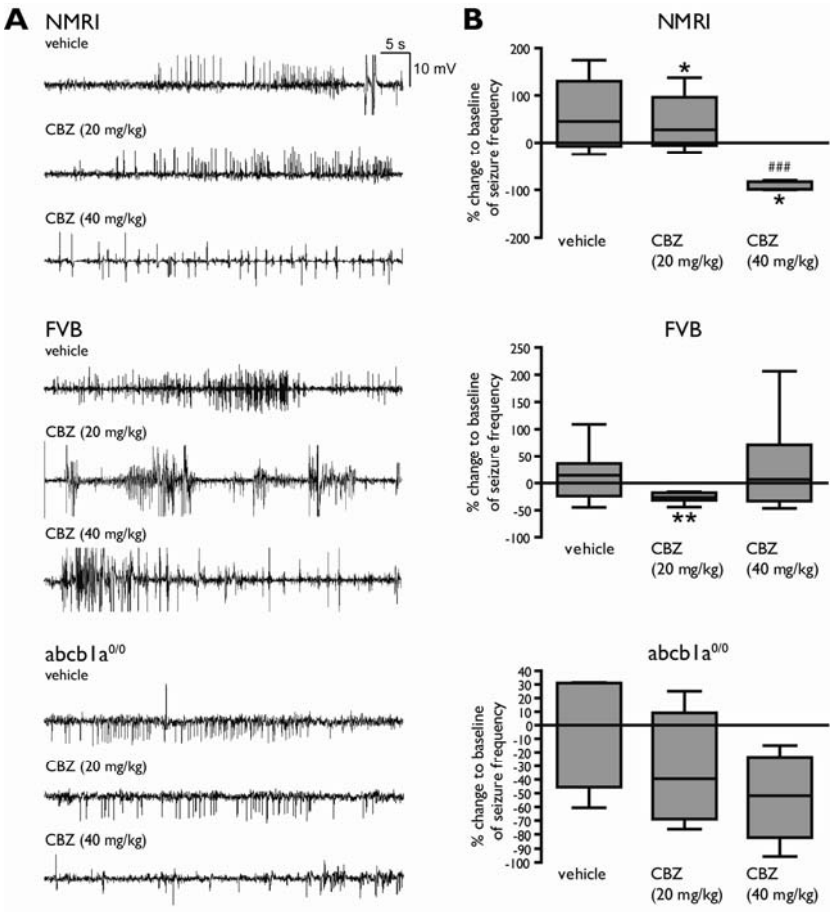

Figure 5.3: Analysis of EEG traces to quantify the antiepileptic action of CBZ in NMRI, FVB and abcb1a$^{0/0}$ mice. ***A:*** *Representative EEG traces for all strains after treatment with vehicle or CBZ (20 mg/kg and 40 mg/kg respectively); in each case, the three traces are from the same animal. Seizure frequency and duration varied inter-individually and intra-individually on different days of recording and no significant strain differences could be detected. In NMRI mice frequent interictal spikes were observed after injection of 40 mg/kg of CBZ. In the FVB strain (WT and mutant), high frequency interictal spiking, especially after injection of 20 mg/kg of CBZ, was evident.* ***B:*** *Quantification of seizure frequency changes. Baseline frequency, before injection, was defined as 100%. Box plots represent the %-changes of seizure frequency after injection compared to the baseline. Significant differences in seizure frequency compared to vehicle injection could not be found, except for NMRI mice. (post-hoc Bonferroni, $^{###}P > 0.001$). Compared to baseline, the seizure frequencies were significantly changed in NMRI mice after injection of 20 mg/kg and 40 mg/kg of CBZ as well as in FVB mice after injection of 20 mg/kg of CBZ (Wilcoxon, $*P < 0.05$, $**P < 0.01$) The number of mice evaluated for each condition ranged from four to seven.*

5.4.3 Seizure suppression by carbamazepine in abcb1a$^{0/0}$ and wild type mice

Following i.p. injection of vehicle, no significant difference in seizure frequency or duration was observed, indicating that the stress of handling and injection did not influence the occurrence of seizures (*Figure 5.3 & Figure 5.4*). Administration of a

therapeutic dose of CBZ (20 mg/kg, i.p.) which elicits acute sedation in NMRI, FVB, and abcb1a$^{0/0}$ mice, failed to significantly suppress seizure activity in NMRI and abcb1a$^{0/0}$ mice, compared to either vehicle or baseline (post-hoc Bonferroni, and Wilcoxon signed rank test) (*Figure 5.3 B*). Unexpectedly, a slight exacerbation of seizures was detected in NMRI mice (+39.6% ± 54.4% compared to baseline; Wilcoxon signed rank test, $P < 0.05$) which was seen in four out of six mice (*Figure 5.4*). FVB mice exhibited a modest reduction in seizure frequency compared to baseline or maintained the same level (*Figure 5.4*), (on average -27.1% ± 9.5%, Wilcoxon signed rank test, $P = 0.0078$*, Figure 5.3 B*). However, compared to vehicle, the difference was not significant (post-hoc Bonferroni) due to variability of seizure frequency between recording sessions.

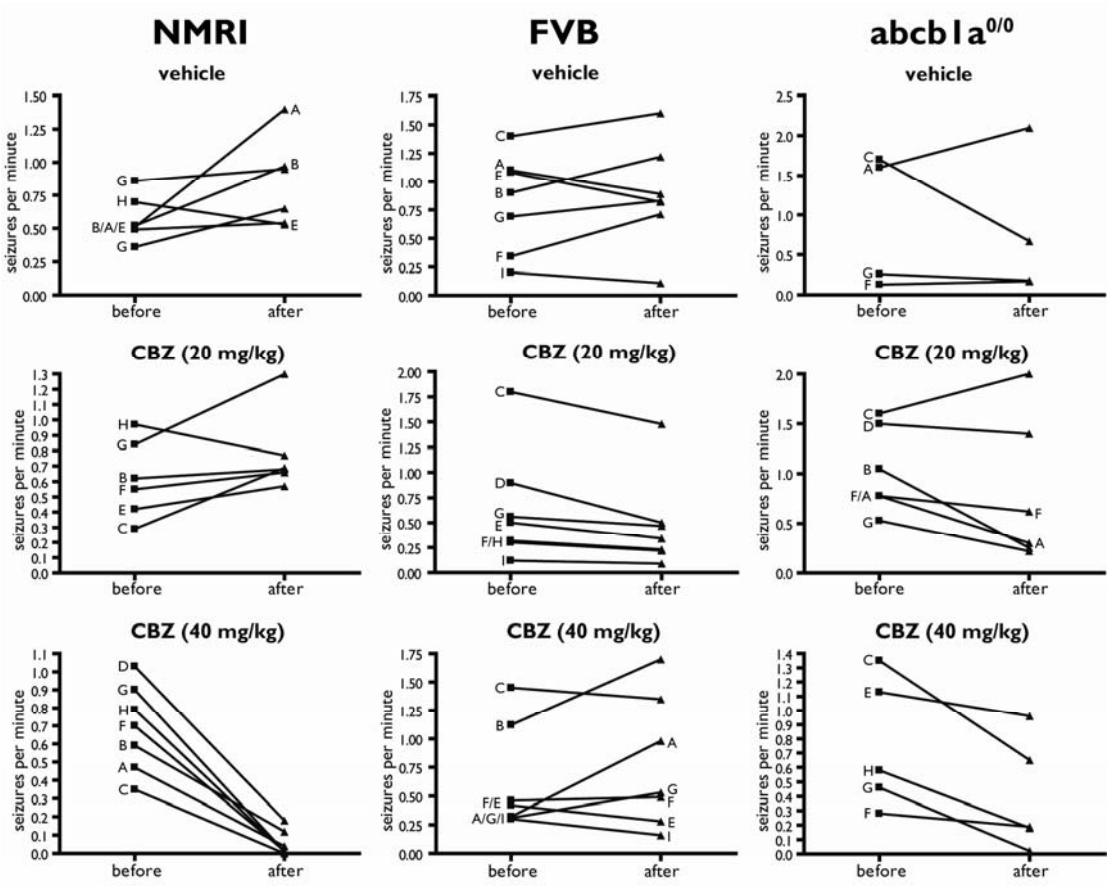

Figure 5.4: Representations of individual seizure frequency changes after CBZ treatment. *Capitalised letters represent individual mice. The seizure frequencies before and after injection of vehicle or CBZ are depicted for each mouse. Thus, frequency changes upon treatment can be seen. Frequencies before injection were obtained on the same day as after injection and served as baseline.*

Finally, analysis of seizure duration revealed no effect of treatment or genotype (two-way ANOVA, not significant) when compared to baseline or to vehicle (*Table 5-1*). Following the injection of a higher dose of CBZ (40 mg/kg), which induced prolonged sedation in all mice, a trend to a genotype effect (two-way ANOVA, $F_{2,46} = 3.015$, $P = 0.0588$) and a treatment effect on seizure frequency was observed ($F_{2,46} = 5.440$, $P = 0.0076$). Thus, seizure suppression was observed in NMRI mice with a decrease of 92.8% $\pm$ 8.5% compared to baseline (Wilcoxon signed rank test, $P = 0.0156$). This finding was confirmed by the reduction in seizure frequency induced by CBZ compared to vehicle (*Figure 5.4*) and post-hoc Bonferroni analysis ($P < 0.001$; *Figure 5.3 B*). Strikingly, these mice displayed frequent interictal spikes and bursts which, however, seldom evolved into seizures. FVB mice showed no significant change in seizure frequency at this dose; however, analysis of EEG traces also revealed a marked increase in the frequency and amplitude of interictal spikes (*Figure 5.3 A*), suggesting that CBZ alters network activity without suppressing seizures. In abcb1a$^{0/0}$ mice, CBZ treatment resulted in a consistent (*Figure 5.4*) but moderate decrease in seizure frequency compared to baseline. While observed in 4 out of 5 mice, the effect was statistically not significant (Wilcoxon signed rank test, $P = 0.0625$); likewise, no significant difference compared to vehicle injection could be detected (*Figure 5.3 B*). Analysis of seizure duration revealed no effect of CBZ irrespective of the genotype (*Table 5-1*), indicating that the drug does not influence a seizure once it has started.
Altogether, the results indicate that CBZ modestly influences either ictal or interictal activity in the three strains of mice analysed. However, no clear involvement of Abcb1a expression could be observed in relation to AED resistance.

5.5 Discussion

The present results demonstrate that the absence of either Abcb1a or Abcc1 does not influence the sedative side-effects of CBZ compared to the parental FVB strain, which lacks detectable Abcc2-immunoreactivity at the BBB (Soontornmalai et al., 2006). However, at low dose, CBZ has stronger sedative action in all lines derived from the FVB strain than in C57BL/6J mice, suggesting that the lack of Abcc2 expression in the BBB might underscore this effect, although the contribution of other genetic differences between FVB and C57BL/6J mice cannot be excluded. We also show that despite its prominent sedative effect in healthy and KA-treated mice, CBZ displays clear strain-specific differences in its antiepileptic action. At the highest dose tested, NMRI mice exhibited a marked reduction of seizure frequency compared to baseline and to vehicle. In contrast, CBZ was ineffective against seizures in FVB WT mice and produced only a minor reduction in abcb1a$^{0/0}$ mutants. Altogether, these results suggest that lack of brain penetration of CBZ does not explain pharmacoresistance in this model of TLE.

5.5.1 Differential sedative effects of carbamazepine

To test whether multidrug transporters limit brain uptake of CBZ under normal physiological conditions, the sedative side effect of CBZ was measured in a test of general activity and locomotion. Both parameters were assessed to ensure that the reduction of locomotion were not due to CBZ-induced ataxia. Upon treatment with vehicle or low CBZ dose (5 mg/kg), mice showed an initial phase of hyperactivity followed by a phase of reduced locomotion, which appeared faster and was significantly stronger after CBZ than vehicle. At the higher CBZ dose (10 mg/kg), sedation was already apparent after 5 minutes, pointing to a rapid onset. Dose-dependence was further evidenced by the profound sedation observed during the recording of EEG activity in epileptic mice treated with 20 mg/kg and 40 mg/kg.

The effects of CBZ did not differ from vehicle after about 60 minutes, because mice show little spontaneous activity during the light (i.e. resting) circadian phase. Our results confirm previous studies reporting a dose-dependent reduction of general and/or locomotor activity following acute CBZ injection in NMRI mice (Redrobe and Bourin, 1999, Li et al., 2004). So far, however, no strain dependence of CBZ effects has been reported. Here, we show that FVB, abcb1a$^{0/0}$, and abcc1$^{0/0}$ mice are more sensitive than C57BL/6J mice, which were sedated only at the higher dose of CBZ.

This effect is not due to an overall difference in motor activity between C57BL/6J and FVB mice, as the response to vehicle-injection was the same in the four lines tested. Most importantly, no difference was seen between FVB mice and abcb1a$^{0/0}$ or abcc1$^{0/0}$ mice. Therefore, the latter two transporters do not appear to significantly limit brain penetration of CBZ under physiological conditions. This conclusion extends previous pharmacological studies carried out in abcb1a$^{0/0}$ and FVB WT mice, which reported no significant difference in serum and brain concentration of CBZ between the two genotypes (Owen et al., 2001a, Sills et al., 2002).

5.5.2 Development of TLE upon KA injection is similar in all strains

To study whether Abcb1a is involved in pharmacoresistance against CBZ, FVB and abcb1a$^{0/0}$ mice were compared here to NMRI mice. As a prerequisite to this study, we verified that epileptogenesis and recurrent seizures are not different in mutant compared to WT mice. No differences among genotypes were observed in the response to KA injection after 4 – 5 weeks post-injection. The morphological changes in the three genotypes were similar and corresponded to previous findings (Bouilleret et al., 1999, Knuesel et al., 2001, Riban et al., 2002, Gouder et al., 2003, Arabadzisz et al., 2005). EEG recordings performed 3 weeks after KA injection also showed that all genotypes have spontaneous recurrent seizures with similar frequency and duration. FVB mice displayed, however, a higher inter-individual variability in seizure frequency with > 6 fold difference between the two extremes. Despite this fact, this mouse model appears well suited to study the role of multidrug transporters for pharmacoresistance in TLE. Furthermore, our observations confirm that abcb1a$^{0/0}$ mice have no apparent phenotype when they are not exposed systemically to potentially toxic substances (Schinkel et al., 1994). Although the BBB is disrupted locally during KA injection, this lesion is not aggravated by the general absence of Abcb1a (or Abcc2). Hence, these transporters are not involved in epileptogenesis *per se*.

5.5.3 Does Abcb1a contribute to resistance against CBZ?

Three weeks after intrahippocampal KA injection, during the chronic phase, CBZ (20 or 40 mg/kg) or vehicle was administered during the EEG recording session to directly assess drug effect on seizure frequency. Despite the strong sedation induced by CBZ

in epileptic mice in the three genotypes tested, they all displayed pharmacoresistance at least to some degree. Unexpectedly, FVB mice were more sensitive to the sedative action of CBZ but more resistant to its antiepileptic action. These observations suggest that the mechanisms responsible for sedation and drug resistance are distinct. Further support for this concept comes from the observation that diazepam, acting as allosteric modulator of $GABA_A$ receptors, has a potent anti-epileptic effect in the KA mouse model of TLE at doses that produce no sedation (Riban et al., 2002). Thus, the sedative action of CBZ and diazepam are likely distinct.

Due to the high inter-and intra-individual variability in seizure frequency, our primary measure of CBZ action was a change in seizure frequency compared to baseline. Administration of the 20 mg/kg dose of CBZ yielded contrasting effects in NMRI and FVB mice, with an exacerbation and a reduction of seizures, respectively. At 40 mg/kg, a clear antiepileptic action was observed in NMRI mice, with seizures being replaced by increased interictal activity whereas FVB WT were unresponsive and abcb1$^{0/0}$ exhibiting a reduction in 4/5 mice. In previous studies, no effect of CBZ was observed in NMRI mice up to 30 mg/kg (Riban et al., 2002, Gouder et al., 2003), suggesting that a very high dose of CBZ, made possible by the formulation used here, is required for suppressing seizures in this TLE model. Finally, the fact that no differences in seizure duration were found between genotypes before and after CBZ injection demonstrates that the drug does not influence a seizure once it has started.

The resistance of FVB mice to CBZ again suggests that the genetic background plays a larger role than the possible absence of Abcc2 from the BBB in regulating seizure activity. Brain penetration of CBZ is evidenced by the increase of interictal activity seen in all strains upon treatment with 40 mg/kg CBZ. Nevertheless, in view of the fact that CBZ reduced seizure frequency compared to baseline in 4 out of 5 abcb1a$^{0/0}$ mice but none of the FVB WT mice suggests a possible contribution of Abcc1a to this effect. A similar trend was evident at the 20 mg/kg dose, with 3 out of 6 mutant mice exhibiting a reduction in seizure frequency. These results are supported by the observation that co-administration of the non-selective Abcb1 inhibitor verapamil in the pilocarpine rat model of TLE significantly potentiates the anticonvulsant activity of oxcarbazepine (Clinckers et al., 2005).

Evidence for a contribution of ABC transporters of the BBB to pharmacoresistance in epilepsy mainly stems from human studies and from in vitro measurements with human ABC transporters. The present results, suggesting a modest contribution, at best of Abcb1a to CBZ pharmacoresistance might be due to species differences in

substrate recognition and transport, or to functional differences between human TLE and the KA-mouse model.

5.5.4 Conclusions

The present results reveal a possible contribution of Abcb1a to resistance against the anti-epileptic effects of CBZ in the KA mouse model, despite the fact that no contribution of this transporter to the sedative effects of CBZ could be detected. Changes in expression and/or function of ABC transporters might selectively occur in the epileptic focus, leading to enhanced function under pathological conditions. However, no clear involvement of an ABC transporter in pharmacoresistance to CBZ could be demonstrated here or in previous *in vivo* or *in vitro* work (Owen et al., 2001a, Baltes et al., 2006). Therefore, additional mechanisms must be involved in pharmacoresistance.

5.6 Acknowledgements

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.
This work was supported by the Swiss National Science Foundation (SNF) NCCR "Neural plasticity and repair" and by grants of the Neuroscience Center Zurich; EMDO-Foundation; Hartmann Müller-Foundation; and Roche Research Foundation.

5.7 Authors' contributions

***Baldinger, M.H.:** Conduction of experiments in epileptic animals including KA injections and electrode implantations, evaluation of EEG data and morphology, statistical analyses, literature research, manuscript preparation and editing. ***Soontornmalai, A.:** Design of the study, conduction of behaviour experiments and evaluation, statistical analyses, literature research, manuscript preparation. **Crestani, F.:** Design of behaviour experiments, statistical analyses, critical reading of manuscript, advisory input. **Fritschy, J.-M.:** Head of research group, design of the study, definition of intellectual content, surveillance of project, advisory input, editing of manuscript.

5.8 References:

See reference section, page 9898.

* contributed equally

6 General discussion

6.1 Résumé

With this work, we aimed to assess the validity of the hypothesis postulating that over-expression of multidrug transporters at the BBB are the main contributors to pharmacoresistance against AEDs. We have performed two independent studies to investigate the substrate specificities of the ABC transporters ABCB1 and ABCC1 for AEDs, and the *in vivo* role of Abcb1 for limited CBZ access to the brain.

In the *in vitro* study, human ABCB1 and ABCC1 were inducibly expressed in MDCK cells and their capacity to transport either of the AEDs lamotrigine and CBZ across a cell monolayer was analysed by LC-MS/MS in a two-compartment cell culture system. As described extensively in chapter 4, we could not demonstrate that either drug is a substrate of ABCB1 or ABCC1. However, we observed a potent endogenous active transport system for these drugs, which could have masked a weak transport mediated by the recombinant proteins.

The second study was performed *in vivo*. Wild type and transporter deficient mice were injected intrahippocampally with KA to induce pharmacoresistant TLE. The impact of transporter deficiency on CBZ sensitivity was assessed by analysing seizure numbers upon injection with CBZ in the chronic phase of TLE. A previous study from our lab (Soontornmalai, 2006) on the sedative effect of low doses of CBZ in healthy mice found no enhanced sedation due to lack of Abcb1a or Abcc1. However, with the lowest dose, a strain difference between C57BL/6 and mice of the FVB background was observed. This could be due to the lack of Abcc2 in the BBB of the FVB background. As elaborated in chapter 5, a strong sedative side effect of higher doses of CBZ was observed also in epileptic mice of all tested genotypes, confirming the previous data. Nevertheless, in respect to treatment of epilepsy with CBZ we observed strain differences as well; however, we were not able to clearly allocate this effect to the presence or absence of Abcb1a. Strikingly, an effect of CBZ on epileptic activity was observed in all strains to some extent. Hence, no marked limitation of CBZ uptake into the brain was detected. EEG patterns after CBZ treatment revealed more interictal spiking than after vehicle treatment, especially in NMRI mice which also displayed less seizures upon injection with a high dose of CBZ. These strain-specific differences in CBZ action, as well as strain-specific expression patterns of ABC transporters are possibly connected. Therefore pharmacoresistance in the KA mouse

model may have something to do with multidrug transporter expression in the BBB or BCSFB to some extent. Taken together, these results showed a very strong sedative action of CBZ in mice, which seems stronger than in human patients. The sedative action seems to arise already at very low doses of the drug, whereas the seizure suppressive action, as seen in the NMRI mice, was only evoked at a rather high dose. Yet, despite the seizure number being markedly reduced in these mice, their EEG pattern did not resemble one of a healthy mouse as demonstrated by Arabadzisz et al. (2005), but rather displays a lot of interictal spiking. To sum up, CBZ has an effect on the healthy brain as well as on the epileptic brain. The question remains, whether the evoked interictal spiking activity, was observed at high doses of the drug, is beneficial or rather detrimental. Additionally, it is not clear if this effect is also seen in human patients or if it is characteristic for the mouse model.

6.2 Methodological considerations

6.2.1 Cell culture study:

We used MDCK cells which were transfected with a vector containing an expression cassette to transcribe an ABC transporter and eGFP under the control of a bi-directional, inducible promoter. The great advantage of an inducible expression system was that the same cell clone, from the same aliquot with the same number of passages could be used as baseline control, and to study the effect of transporter expression. This ensured that expression of endogenous proteins was the same in both conditions. Therefore, baseline active transport and passive diffusion was in the same range, leading to reliable, robust results with little variations. Potential diverging differentiation of sub-clones due to separate culturing could be ruled out. Hence, the best possible baseline was achieved, thus providing for high reproducibility of the results. Therefore the BBB model we developed is well suited to conduct AED transport studies.

Despite the generation of monoclonal cell lines, we observed, upon induction of gene-expression with DOX, a mosaic-like expression of eGFP as well as ABC transporters which was not congruent. Hence, the co-expression of eGFP could not be used as quantitative marker for transporter expression as originally planned, but only to check for induced gene-expression upon addition of DOX. Retrospectively, it might have been better not to use such a bi-directional expression system as the benefit from eGFP expression was only minor and the over-expression of eGFP in addition to the

transporter could have unnecessarily stressed the cells. Cloning of the constructs was also quite difficult and may have been easier without the bi-directional promoter and eGFP.

No transport of either lamotrigine or CBZ was found, however, a weak effect could have been overlooked. In this line it has to be considered that we can not be certain that all transporters are fully functional despite proper localisation. By over-expression a lot more transporters are produced than would be in a physiological surrounding. Therefore, a limitation of necessary co-factors for individual transporters could arise due to titration of the supplies. In this case, weak transport by only few functional transporters may be missed. Another possibility are missing interaction partners or signals which would lead to functional changes e.g. caused by a pathology, which in turn would allow efficient drug-extrusion by ABCB1 or ABCC1. Furthermore it has to be noted that transport was only measured over a limited surface area of 0.33 cm^2 in our case, whereas the surface area of the murine BBB is much larger, hence, also a weak effect, which might be missed in the experimental setup, can have an impact *in vivo*.

MDCK cells were chosen as they form tight monolayers which are easy to cultivate and transfect and have been used before in similar drug-transport studies. While being well suited for such applications, a few disadvantages have to be kept in mind. Due to their origin (from the kidney nephron) and original function (extrusion of xenobiotics and toxins), they express a vast array of endogenous transporters (Goh et al., 2002). The exact expression pattern is, to our knowledge, not completely characterised. The expression of endogenous transporters may be problematic as they might be more efficient in transporting a compound to be tested than the recombinantly expressed protein, thereby masking effects of the latter. Nevertheless, using another cell line would not have solved this problem. The approach to use immortalised brain endothelial cells was not suitable in our experiments as the available endothelial cell lines do not display high enough impermeability mediated by tight junctions which is fundamental for the assessment of transcellular transport (Deli et al., 2005). The use of primary endothelial cells was no option either due to two reasons. On one hand, they cannot be cultivated over prolonged periods of time, which is essential for stable transfections followed by transport studies, and on the other hand, only grow to tight enough monolayers when cultivated in contact with astrocytes (Deli et al., 2005). However, this would impede with the transport studies, as two cell layers would have to be crossed. Additionally, all cells which are suitable to

perform transport studies come from tissue which is involved in selective uptake or extrusion processes and therefore express a range of endogenous transporters. So far, the only way to overcome this problem is to use inhibitors which are reasonably specific. This stresses again the need for a good baseline control, which our system could offer.

6.2.2 Animal study:

The intrahippocampal KA mouse model of TLE led to robust results. If the correct amount of KA was injected into the right location of the brain the lesion and chronic seizures in all mice were comparable. Thus, it is a good system to study epilepsy treatment across genotypes.

Nevertheless, there are some concerns also with this system. The main one is, as with all animal models of diseases, the validity. To what extent does an animal model mimic the real phenotype found in humans? One can only speculate, and try to work with the best system available.

There are some technical difficulties which had to be considered. Not all animals which had been operated could be analysed due to loss of electrodes before or during the experiment or to absence of or bad EEG signals because of faulty electrodes or absence of seizures due to misplaced KA injection. We aimed to measure at least 5 animals per genotype and condition to have a high enough n for statistical analyses. According to our experience it was thus necessary to operate three times more animals than the finally desired number, in our case at least 15 mice per genotype.

A somewhat unexpected problem was the size of the abcb1a$^{0/0}$ mice. So far this mouse line has not been characterised extensively. It was therefore surprising to find that most mice of this strain barely weighed 20 g, even after 4 – 6 months of age. This complicated the experiments as the mice reacted much more sensitive to the KA injection, sometimes even displaying convulsive SE after injection of the correct amount of KA. Also FVB mice were more sensitive to KA and the mortality after anaesthesia and during SE was rather high compared to mice of the NMRI strain.

Additionally, abcb1a$^{0/0}$ mice displayed hyperactivity compared to WT mice. This had two implications: They tended to lose their electrodes more frequently than the WT and had more moving artefacts during EEG recordings, especially after vehicle injection. It was also observed that the transporter-deficient mice were difficult to breed. They displayed seasonal variations and generally only small litters.

For best results in the EEG recordings, we tried to minimise moving artefacts by surveying the animals during the light period (resting phase of the mice) and without food or water in the chambers. Nevertheless, artefacts could not completely be avoided. A time frame with as little artefacts as possible, but not too long after drug-injection, was defined for each animal and condition, to evaluate seizure number and duration. Depending on the localisation of the bipolar electrodes in respect to the epileptic focus the amplitudes of seizures were varying and in many cases getting lower over time. These challenges may also be the reason that not many quantitative studies of seizure analysis have been published so far. To improve EEG recordings and reduce moving artefacts based on movement of the cables, it would be best to use a telemetry system. This would also allow 24 h surveillance. However, at the timepoint when this study was started, such a system was not available in the lab.

6.3 Are the results from the two studies comparable?

The combination of assays in a cell culture system with inducible over-expression of human transporters and analysis in WT and transporter-deficient animals was used to test two fundamental properties, to give a more global picture of transporter function. i) Are the drugs substrates of ABC transporters; and ii) Are transporter-deficient mice more sensitive to drug treatment than WT mice? The advantages and disadvantages of the two approaches were thought to complement and balance each other out. While *in vitro* assays lack some of the complex interactions which would happen in the physiological context, the opposite argument can be used against *in vivo* studies. As observed in our study, there may be marked strain differences which make the interpretation of a result complicated. The complexity of a whole organism is problematic as it is difficult to assign a specific phenotype to a single mechanism or molecule. Let alone the question as to what extent an animal model really represents a human disease and the findings can be applied to the disease. If the two studies would have a clear, 'yes or no', outcome, the interpretation would be easy. However, the *in vitro* work seems to rule out an involvement of human ABC transporters in AED resistance whereas the results from the *in vivo* work do not completely. This could be explained by the possibility that the transporters from mice and men do not have the same substrate specificity. This has been speculated before and was assessed by

Baltes et al. (2006). It has to be noted that the candidate which seems most promising form the *in vivo* studies, Abcc2, was not tested *in vitro* so far.

Another possible explanation which could partly explain the discrepancies between the two studies is the hypothesis that substrate specificity may be regulated by the pathology. In this case our results would argue against an onset of pharmacoresistance in TLE upon upregulation of transporter expression as suggested by Dombrowski et al. (2001) and Kubota et al. (2006) but rather for functional changes, possibly through post-translational modifications, which could lead to enhanced transport of AEDs upon onset of TLE.

However, to our knowledge, for both studies the best available, reliable systems were used. Hence, the fact that no clear involvement in drug export of either transporter could be demonstrated *in vitro* or *in vivo*, hints that the tested transporters are not, or only to a minor degree involved in resistance to the drugs tested. In this respect the two approaches are comparable and complement each other.

6.4 Validity of the multidrug transporter hypothesis of pharmacoresistance in TLE

From the results of this work it seems unlikely that ABCB1 and ABCC1 are involved to resistance to the AEDs lamotrigine and CBZ. The possibility of masked transport through other means in the *in vitro* study and the involvement of transporter-deficiency on the genotype-specific effect of CBZ *in vivo* point to minor involvement at most. To proof that a specific transporter is responsible for the transport of a certain drug, a strong effect *in vitro* and *in vivo* would be expected. Evidence, supporting our findings, which rules an involvement of ABC transporters in AED resistance out, is accumulating. While Owen et al. (2001) and Potschka et al. (2003) could not demonstrate involvement of ABCB1 or Abcc2 in resistance to CBZ and lamotrigine respectively, Cerveny et al. (2006) could not demonstrate ABCG2 involvement either. Baltes et al. (2007) showed that neither ABCB1, ABCC1 nor ABCC2 are not involved in transport of valproic acid. Nevertheless, proteins from other families could be the main players and a concerted action of several transporters may possibly occur at the BBB and also the BCSFB. One candidate is the non-ABC multi-specific transporter RLIP76 which has been shown to transport the two AEDs phenytoin and CBZ (Awasthi et al., 2005). However, all together, it seems that the transporter hypothesis of AED resistance in TLE had been emphasised too strongly in the past. Most probably, AED

extrusion by ABC or other transporters is not the main and certainly not the only mechanism. The facts that AED resistance includes many different compounds and most TLE patients are affected but not all, suggest that several resistance mechanisms act individually or in concert and may vary from case to case. Some evidence for all hypotheses which have been put forward so far, the transporter hypothesis, the enzyme hypothesis and the drug-target hypothesis, has been published. While researchers try to verify their hypothesis, looking for one effect, they may miss others, especially in animal models. Hence, multiple mechanisms acting together to sustain pharmacoresistance could also mask effects of transporter depletion. Therefore no clear 'yes or no' answers could be found, also in our work. It seems naïve to believe that all possible mechanisms have been identified. Some mechanisms contributing to pharmacoresistance of one or another compound in one or another patient may still remain to be discovered.

Several polymorphisms are known for ABC transporters and it has been demonstrated that these may display functional differences e.g. in substrate recognition (Kimchi-Sarfaty et al., 2007). It is therefore possible that the cDNA of the allele we used for our recombinant expression system is less sensitive to CBZ and lamotrigine than another one would be. In this respect it might be interesting to analyse the genotypes of patients with or without pharmacoresistance. In recent years, several groups have tried to address the impact of pathology induced genomic responses, i.e. changes of gene expression profiles, using gene chip based molecular profiling of tissue from TLE patients or animal models (Majores et al., 2004). Others have tried to assign certain polymorphisms to TLE, however, most of this data has yet to be replicated (Cavalleri et al., 2005). Besides, so far, most associations of polymorphisms in patients or changes of gene expression were made in respect to pathogenesis and data in respect to pharmacoresistance is largely lacking, except for data supporting the transporter hypothesis on ABCB1 (Zimprich et al., 2004). Based on the finding that this hypothesis is not the most important for pharmacoresistance, genotype analysis should be directed towards other candidates in the future.

6.5 Future directions

Epilepsy is still a major neurological disorder of which only little is understood. To improve the quality of life of patients, the development of better tolerable treatments with fewer side effects is necessary. Generally, in depth understanding of all different types of epilepsy will be necessary for future development of therapies or even cures. Therefore, it has been and will also be necessary in the future to combine various approaches of research: animal models, molecular approaches *in vitro*, and analysis of data from patients. Together, this will hopefully lead to the design of a new generation of effective treatments.

In respect to the transporter hypothesis of pharmacoresistance in TLE additional experiments could be carried out to identify the key players. Both systems used in this work can be further developed and modulated. With the intrahippocampal KA mouse model of TLE, it would also be possible to study the effect of other AEDs on seizures in wild type and transporter deficient mice, also of other genotypes. With the existing cell culture system it would be easy to test for transport of other AEDs and further cell lines could be generated, expressing other candidates for AED transport, such as ABCG2 (BCRP), OATs or RLIP76.

To overcome the problem of effects by unknown endogenous transporters it may be necessary to establish totally novel cell culture systems, using cells which lack endogenous transporters. Such cells may have the downside not to form tight junctions. In this case it may be possible to transfect them not only with the transporter of interest, but additionally with all genes needed for the generation of tight junctions, thus creating cells with can build tight monolayers while expressing only the transporter of interest.

With respect to the fact that the transporter hypothesis is probably not the main factor leading to AED resistance, the main focus of future research should lie on the examination of other mechanisms. This, especially in respect to future drug-development, to find compounds with better efficacy and fewer side effects. The recent approaches to use co-treatment of AEDs with transporter-inhibitors seems not a good option due to strong side effects of the inhibitors and little benefit, especially since the effect of the transporters may not be as pronounced as previously thought. The impact of enzymes at the BBB or BCSFB on AED resistance will have to be investigated as well. If enzymes are the key players, their blockage might be a

possible strategy for treatment. However, side effects would also be an issue of concern in this case.

Pharmacoresistance is likely to underlie transcriptional and post-transcriptional regulations or functional changes occurring upon development of TLE and seizures. Hence, an important direction of research must lie in the understanding of functional alterations of drug targets due to genetic variations or pathological changes. In this respect genotype analysis of healthy subjects and epileptic patients may reveal new insights on the pathology itself as well as the underlying pharmacoresistance. If novel candidates are defined in such an approach, these can then be further investigated as to what their involvement is in pharmacoresistance and by which mechanisms they act. Based on this, it should then be possible to develop novel drugs with different modes of actions or targets and to which the patients will be responsive.

7 References

Alan Peters, S. L. P., Webster, H. deF., 1991. The fine structure of the nervous system: Neurons and their supporting cells. Oxford Press.

Albani, F., Riva, R. and Baruzzi, A., 1995. Carbamazepine clinical pharmacology: a review. Pharmacopsychiatry. 28, 235-244.

Ambrosio, A. F., Silva, A. P., Malva, J. O., Soares-da-Silva, P., Carvalho, A. P. and Carvalho, C. M., 2001. Inhibition of glutamate release by BIA 2-093 and BIA 2-024, two novel derivatives of carbamazepine, due to blockade of sodium but not calcium channels. Biochem Pharmacol. 61, 1271-1275.

Arabadzisz, D., Antal, K., Parpan, F., Emri, Z. and Fritschy, J. M., 2005. Epileptogenesis and chronic seizures in a mouse model of temporal lobe epilepsy are associated with distinct EEG patterns and selective neurochemical alterations in the contralateral hippocampus. Exp Neurol. 194, 76-90.

Aronica, E., Gorter, J. A., Jansen, G. H., van Veelen, C. W., van Rijen, P. C., Leenstra, S., Ramkema, M., Scheffer, G. L., Scheper, R. J. and Troost, D., 2003. Expression and cellular distribution of multidrug transporter proteins in two major causes of medically intractable epilepsy: focal cortical dysplasia and glioneuronal tumors. Neuroscience. 118, 417-429.

Aronica, E., Gorter, J. A., Ramkema, M., Redeker, S., Ozbas-Gerceker, F., van Vliet, E. A., Scheffer, G. L., Scheper, R. J., van der Valk, P., Baayen, J. C. and Troost, D., 2004. Expression and cellular distribution of multidrug resistance-related proteins in the hippocampus of patients with mesial temporal lobe epilepsy. Epilepsia. 45, 441-451.

Aronica, E., Gorter, J. A., Redeker, S., van Vliet, E. A., Ramkema, M., Scheffer, G. L., Scheper, R. J., van der Valk, P., Leenstra, S., Baayen, J. C., Spliet, W. G. and Troost, D., 2005. Localization of breast cancer resistance protein (BCRP) in microvessel endothelium of human control and epileptic brain. Epilepsia. 46, 849-857.

Avanzini, G. and Franceschetti, S., 2003. Cellular biology of epileptogenesis. Lancet Neurol. 2, 33-42.

Awasthi, S., Hallene, K. L., Fazio, V., Singhal, S. S., Cucullo, L., Awasthi, Y. C., Dini, G. and Janigro, D., 2005. RLIP76, a non-ABC transporter, and drug resistance in epilepsy. BMC Neurosci. 6, 61.

Baltes, S., Fedrowitz, M., Tortos, C. L., Potschka, H. and Loscher, W., 2007. Valproic acid is not a substrate for P-glycoprotein or multidrug resistance proteins 1 and 2 in a number of in vitro and in vivo transport assays. J Pharmacol Exp Ther. 320, 331-343.

Baltes, S., Gastens, A. M., Fedrowitz, M., Potschka, H., Kaever, V. and Loscher, W., 2006. Differences in the transport of the antiepileptic drugs phenytoin, levetiracetam and carbamazepine by human and mouse P-glycoprotein. Neuropharmacology.

Boison, D., 2008. The adenosine kinase hypothesis of epileptogenesis. Prog Neurobiol. 84, 249-262.

Bouilleret, V., Ridoux, V., Depaulis, A., Marescaux, C., Nehlig, A. and Le Gal La Salle, G., 1999. Recurrent seizures and hippocampal sclerosis following intrahippocampal kainate injection in adult mice: electroencephalography, histopathology and synaptic reorganization similar to mesial temporal lobe epilepsy. Neuroscience. 89, 717-729.

Bouilleret, V., Schwaller, B., Schurmans, S., Celio, M. R. and Fritschy, J. M., 2000. Neurodegenerative and morphogenic changes in a mouse model of temporal lobe epilepsy do not depend on the expression of the calcium-binding proteins parvalbumin, calbindin, or calretinin. Neuroscience. 97, 47-58.

Bourgeois, B., 2001. Pharmacokinetics and pharmacodynamics of antiepileptic drugs. In: Wyllie, E. (Ed.), The treatment of epilepsy: Principles and practice. Lippincott Williams and Wilkins.

Browne, T. R., Holmes, G. L., 2001. Epilepsy. N Engl J Med. 344, 1145-1151.

Burckhardt, B. C. and Burckhardt, G., 2003. Transport of organic anions across the basolateral membrane of proximal tubule cells. Rev Physiol Biochem Pharmacol. 146, 95-158.

Cavalheiro, E. A., Naffah-Mazzacoratti, M.G., Mello, L.E., Leite, J.P., 2006. The pilocarpine model of seizures. In: Pitkanen, A., Schwartzkroin, P.A., Moshe, S.L. (Ed.), Models of seizures and epilepsy. Elsevier Academic Press.

Cavalleri, G. L., Lynch, J. M., Depondt, C., Burley, M. W., Wood, N. W., Sisodiya, S. M. and Goldstein, D. B., 2005. Failure to replicate previously reported genetic associations with sporadic temporal lobe epilepsy: where to from here? Brain. 128, 1832-1840.

Cerveny, L., Pavek, P., Malakova, J., Staud, F. and Fendrich, Z., 2006. Lack of interactions between breast cancer resistance protein (bcrp/abcg2) and selected antiepileptic agents. Epilepsia. 47, 461-468.

Clinckers, R., Smolders, I., Meurs, A., Ebinger, G. and Michotte, Y., 2005. Quantitative in vivo microdialysis study on the influence of multidrug transporters on the blood-brain barrier passage of oxcarbazepine: concomitant use of hippocampal monoamines as pharmacodynamic markers for the anticonvulsant activity. J Pharmacol Exp Ther. 314, 725-731.

Cockerell, O. C., Johnson, A. L., Sander, J. W., Hart, Y. M. and Shorvon, S. D., 1995. Remission of epilepsy: results from the National General Practice Study of Epilepsy. Lancet. 346, 140-144.

Cole, S. P., Bhardwaj, G., Gerlach, J. H., Mackie, J. E., Grant, C. E., Almquist, K. C., Stewart, A. J., Kurz, E. U., Duncan, A. M. and Deeley, R. G., 1992. Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. Science. 258, 1650-1654.

Dawson, R. J. and Locher, K. P., 2006. Structure of a bacterial multidrug ABC transporter. Nature. 443, 180-185.

de Vries, N. A., Zhao, J., Kroon, E., Buckle, T., Beijnen, J. H. and van Tellingen, O., 2007. P-glycoprotein and breast cancer resistance protein: two dominant transporters working together in limiting the brain penetration of topotecan. Clin Cancer Res. 13, 6440-6449.

Deeley, R. G., Westlake, C. and Cole, S. P., 2006. Transmembrane transport of endo- and xenobiotics by mammalian ATP-binding cassette multidrug resistance proteins. Physiol Rev. 86, 849-899.

Delanty, N., French, J. A., 2001. Newer antiepileptic drugs. In: Wyllie, E. (Ed.), The treatment of epilepsy: Principles and practice. Lippincott Williams and Wilkins.

Deli, M. A., Abraham, C. S., Kataoka, Y. and Niwa, M., 2005. Permeability studies on in vitro blood-brain barrier models: physiology, pathology, and pharmacology. Cell Mol Neurobiol. 25, 59-127.

Dombrowski, S. M., Desai, S. Y., Marroni, M., Cucullo, L., Goodrich, K., Bingaman, W., Mayberg, M. R., Bengez, L. and Janigro, D., 2001. Overexpression of multiple drug resistance genes in endothelial cells from patients with refractory epilepsy. Epilepsia. 42, 1501-1506.

Dua, T., de Boer, H. M., Prilipko, L. L. and Saxena, S., 2006. Epilepsy Care in the World: results of an ILAE/IBE/WHO Global Campaign Against Epilepsy survey. Epilepsia. 47, 1225-1231.

Dudek, F. E., Clark, S., Williams, P.A., Grabenstatter, H.L., 2006. Kainate-induced status epilepticus: A chronic model of acquired epilepsy. In: Pitkanen, A., Schwartzkroin, P.A., Moshe, S.L. (Ed.), Models of seizures and epilepsy. Elsevier Academic Press.

Ellerkmann, R. K., Remy, S., Chen, J., Sochivko, D., Elger, C. E., Urban, B. W., Becker, A. and Beck, H., 2003. Molecular and functional changes in voltage-dependent Na(+) channels following pilocarpine-induced status epilepticus in rat dentate granule cells. Neuroscience. 119, 323-333.

Evers, R., de Haas, M., Sparidans, R., Beijnen, J., Wielinga, P. R., Lankelma, J. and Borst, P., 2000a. Vinblastine and sulfinpyrazone export by the multidrug resistance protein MRP2 is associated with glutathione export. Br J Cancer. 83, 375-383.

Evers, R., Kool, M., Smith, A. J., van Deemter, L., de Haas, M. and Borst, P., 2000b. Inhibitory effect of the reversal agents V-104, GF120918 and Pluronic L61 on MDR1 Pgp-, MRP1- and MRP2-mediated transport. Br J Cancer. 83, 366-374.

Fedele, D. E., Gouder, N., Guttinger, M., Gabernet, L., Scheurer, L., Rulicke, T., Crestani, F. and Boison, D., 2005. Astrogliosis in epilepsy leads to overexpression of adenosine kinase, resulting in seizure aggravation. Brain. 128, 2383-2395.

Francis, J. and Burnham, W. M., 1992. [3H]Phenytoin identifies a novel anticonvulsant-binding domain on voltage-dependent sodium channels. Mol Pharmacol. 42, 1097-1103.

French, J. A., Kanner, A. M., Bautista, J., Abou-Khalil, B., Browne, T., Harden, C. L., Theodore, W. H., Bazil, C., Stern, J., Schachter, S. C., Bergen, D., Hirtz, D., Montouris, G. D., Nespeca, M., Gidal, B., Marks, W. J., Jr., Turk, W. R., Fischer, J. H., Bourgeois, B., Wilner, A., Faught, R. E., Jr., Sachdeo, R. C., Beydoun, A. and Glauser, T. A., 2004. Efficacy and tolerability of the new antiepileptic drugs I: treatment of new onset epilepsy: report of the Therapeutics and Technology Assessment Subcommittee and Quality Standards Subcommittee of the American Academy of Neurology and the American Epilepsy Society. Neurology. 62, 1252-1260.

Fujikawa, D. G., 2005. Prolonged seizures and cellular injury: understanding the connection. Epilepsy Behav. 7 Suppl 3, S3-11.

Gallagher, J. P., Higashi, H. and Nishi, S., 1978. Characterization and ionic basis of GABA-induced depolarizations recorded in vitro from cat primary afferent neurones. J Physiol. 275, 263-282.

Gazzin, S., Strazielle, N., Schmitt, C., Fevre-Montange, M., Ostrow, J. D., Tiribelli, C. and Ghersi-Egea, J. F., 2008. Differential expression of the multidrug resistance-related proteins ABCb1 and ABCc1 between blood-brain interfaces. J Comp Neurol. 510, 497-507.

Ghersi-Egea, J. F., Leninger-Muller, B., Suleman, G., Siest, G. and Minn, A., 1994. Localization of drug-metabolizing enzyme activities to blood-brain interfaces and circumventricular organs. J Neurochem. 62, 1089-1096.

Gibbs, J. P., Adeyeye, M. C., Yang, Z. and Shen, D. D., 2004. Valproic acid uptake by bovine brain microvessel endothelial cells: role of active efflux transport. Epilepsy Res. 58, 53-66.

Glauser, T. A., Graves, N. M., 2001. Phenytoin and Fosphenytoin. In: Wyllie, E. (Ed.), The treatment of epilepsy: principles and practice. Lippincott Williams and Wilkins, pp. 853-868.

Goh, L. B., Spears, K. J., Yao, D., Ayrton, A., Morgan, P., Roland Wolf, C. and Friedberg, T., 2002. Endogenous drug transporters in in vitro and in vivo models for the prediction of drug disposition in man. Biochem Pharmacol. 64, 1569-1578.

Gouder, N., Fritschy, J. M. and Boison, D., 2003. Seizure suppression by adenosine A1 receptor activation in a mouse model of pharmacoresistant epilepsy. Epilepsia. 44, 877-885.

Gouder, N., Scheurer, L., Fritschy, J. M. and Boison, D., 2004. Overexpression of adenosine kinase in epileptic hippocampus contributes to epileptogenesis. J Neurosci. 24, 692-701.

Grisar, T., Guillaume, D. and Delgado-Escueta, A. V., 1992. Contribution of Na+,K(+)-ATPase to focal epilepsy: a brief review. Epilepsy Res. 12, 141-149.

Higgins, C. F., 2007. Multiple molecular mechanisms for multidrug resistance transporters. Nature. 446, 749-757.

Hippocrates, ca 400 BC. On the Sacred Disease.

Hollenstein, K., Dawson, R. J. and Locher, K. P., 2007. Structure and mechanism of ABC transporter proteins. Curr Opin Struct Biol. 17, 412-418.

Huisman, M. T., Smit, J. W., Crommentuyn, K. M., Zelcer, N., Wiltshire, H. R., Beijnen, J. H. and Schinkel, A. H., 2002. Multidrug resistance protein 2 (MRP2) transports HIV protease inhibitors, and transport can be enhanced by other drugs. Aids. 16, 2295-2301.

ILAE, 1981. Proposal for revised clinical and electroencephalographic classification of epileptic seizures. From the Commission on Classification and Terminology of the International League Against Epilepsy. Epilepsia. 22, 489-501.

Ito, K., Suzuki, H., Horie, T. and Sugiyama, Y., 2005. Apical/basolateral surface expression of drug transporters and its role in vectorial drug transport. Pharm Res. 22, 1559-1577.

Jandova, K., Pasler, D., Antonio, L. L., Raue, C., Ji, S., Njunting, M., Kann, O., Kovacs, R., Meencke, H. J., Cavalheiro, E. A., Heinemann, U., Gabriel, S. and Lehmann, T. N., 2006. Carbamazepine-resistance in the epileptic dentate gyrus of human hippocampal slices. Brain. 129, 3290-3306.

Janigro, D., Ghosh, D., Marchi, N., Betto, G., 2008. First evidence for carbamazepine metabolism by human blood-brain barrier endothelial cells: implications for anti-epileptic therapy. 311.Epilepsy: Mechanisms and Therapy. Proceedings of the SfN Neuroscience 2008, Washington DC, pp.

Joo, F., 1992. The cerebral microvessels in culture, an update. J Neurochem. 58, 1-17.

Joo, F. and Karnushina, I., 1973. A procedure for the isolation of capillaries from rat brain. Cytobios. 8, 41-48.

Juliano, R. L. and Ling, V., 1976. A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. Biochim Biophys Acta. 455, 152-162.

Kilic, E., Spudich, A., Kilic, U., Rentsch, K. M., Vig, R., Matter, C. M., Wunderli-Allenspach, H., Fritschy, J. M., Bassetti, C. L. and Hermann, D. M., 2008. ABCC1: a gateway for pharmacological compounds to the ischaemic brain. Brain. 131, 2679-2689.

Kimchi-Sarfaty, C., Oh, J. M., Kim, I. W., Sauna, Z. E., Calcagno, A. M., Ambudkar, S. V. and Gottesman, M. M., 2007. A "silent" polymorphism in the MDR1 gene changes substrate specificity. Science. 315, 525-528.

Knuesel, I., Zuellig, R. A., Schaub, M. C. and Fritschy, J. M., 2001. Alterations in dystrophin and utrophin expression parallel the reorganization of GABAergic synapses in a mouse model of temporal lobe epilepsy. Eur J Neurosci. 13, 1113-1124.

Krestel, H. E., Mayford, M., Seeburg, P. H. and Sprengel, R., 2001. A GFP-equipped bidirectional expression module well suited for monitoring tetracycline-regulated gene expression in mouse. Nucleic Acids Res. 29, E39.

Kubota, H., Ishihara, H., Langmann, T., Schmitz, G., Stieger, B., Wieser, H. G., Yonekawa, Y. and Frei, K., 2006. Distribution and functional activity of P-glycoprotein and multidrug resistance-associated proteins in human brain microvascular endothelial cells in hippocampal sclerosis. Epilepsy Res. 68, 213-228.

Kuo, C. C. and Lu, L., 1997. Characterization of lamotrigine inhibition of Na+ channels in rat hippocampal neurones. Br J Pharmacol. 121, 1231-1238.

Kwan, P. and Brodie, M. J., 2004. Phenobarbital for the treatment of epilepsy in the 21st century: a critical review. Epilepsia. 45, 1141-1149.

Kwan, P. and Brodie, M. J., 2005. Potential role of drug transporters in the pathogenesis of medically intractable epilepsy. Epilepsia. 46, 224-235.

Kwan, P., Sills, G. J., Butler, E., Gant, T. W., Meldrum, B. S. and Brodie, M. J., 2002. Regional expression of multidrug resistance genes in genetically epilepsy-prone rat brain after a single audiogenic seizure. Epilepsia. 43, 1318-1323.

Lee, G., Dallas, S., Hong, M. and Bendayan, R., 2001. Drug transporters in the central nervous system: brain barriers and brain parenchyma considerations. Pharmacol Rev. 53, 569-596.

Leppik, I. E., 2004. Zonisamide: chemistry, mechanism of action, and pharmacokinetics. Seizure. 13 Suppl 1, S5-9; discussion S10.

Leslie, E. M., Deeley, R. G. and Cole, S. P., 2001. Toxicological relevance of the multidrug resistance protein 1, MRP1 (ABCC1) and related transporters. Toxicology. 167, 3-23.

Li, J. X., Zhao, W. L. and Liang, J. H., 2004. Effects of carbamazepine on morphine-induced behavioral sensitization in mice. Brain Res. 1019, 77-83.

Loscher, W., 2002. Current status and future directions in the pharmacotherapy of epilepsy. Trends Pharmacol Sci. 23, 113-118.

Loscher, W., 2007. Drug transporters in the epileptic brain. Epilepsia. 48 Suppl 1, 8-13.

Loscher, W. and Potschka, H., 2002. Role of multidrug transporters in pharmacoresistance to antiepileptic drugs. J Pharmacol Exp Ther. 301, 7-14.

Loscher, W. and Potschka, H., 2005. Drug resistance in brain diseases and the role of drug efflux transporters. Nat Rev Neurosci. 6, 591-602.

Loup, F., Wieser, H. G., Yonekawa, Y., Aguzzi, A. and Fritschy, J. M., 2000. Selective alterations in GABAA receptor subtypes in human temporal lobe epilepsy. J Neurosci. 20, 5401-5419.

Luer, M. S., Hamani, C., Dujovny, M., Gidal, B., Cwik, M., Deyo, K. and Fischer, J. H., 1999. Saturable transport of gabapentin at the blood-brain barrier. Neurol Res. 21, 559-562.

Luna-Tortos, C., Fedrowitz, M. and Loscher, W., 2008. Several major antiepileptic drugs are substrates for human P-glycoprotein. Neuropharmacology. 55, 1364-1375.

Lynch, B. A., Lambeng, N., Nocka, K., Kensel-Hammes, P., Bajjalieh, S. M., Matagne, A. and Fuks, B., 2004. The synaptic vesicle protein SV2A is the binding site for the antiepileptic drug levetiracetam. Proc Natl Acad Sci U S A. 101, 9861-9866.

Majores, M., Eils, J., Wiestler, O. D. and Becker, A. J., 2004. Molecular profiling of temporal lobe epilepsy: comparison of data from human tissue samples and animal models. Epilepsy Res. 60, 173-178.

Mao, Q., Deeley, R. G. and Cole, S. P., 2000. Functional reconstitution of substrate transport by purified multidrug resistance protein MRP1 (ABCC1) in phospholipid vesicles. J Biol Chem. 275, 34166-34172.

Marson, A. G., Al-Kharusi, A. M., Alwaidh, M., Appleton, R., Baker, G. A., Chadwick, D. W., Cramp, C., Cockerell, O. C., Cooper, P. N., Doughty, J., Eaton, B., Gamble, C., Goulding, P. J., Howell, S. J., Hughes, A., Jackson, M., Jacoby, A., Kellett, M., Lawson, G. R., Leach, J. P., Nicolaides, P., Roberts, R., Shackley, P., Shen, J., Smith, D. F., Smith, P. E., Smith, C. T., Vanoli, A. and Williamson, P. R., 2007. The SANAD study of effectiveness of carbamazepine, gabapentin, lamotrigine, oxcarbazepine, or topiramate for treatment of partial epilepsy: an unblinded randomised controlled trial. Lancet. 369, 1000-1015.

Mattson, R. H., Cramer, J. A. and Collins, J. F., 1996. Prognosis for total control of complex partial and secondarily generalized tonic clonic seizures. Department of Veterans Affairs Epilepsy Cooperative Studies No. 118 and No. 264 Group. Neurology. 47, 68-76.

McIntyre, D. C., 2006. The kindling phenomenon. In: Pitkanen, A., Schwartzkroin, P.A., Moshe, S.L. (Ed.), Models of Seizures and Epilepsy. Elsevier Academic Press.

Meisler, M. H., Kearney, J., Ottman, R. and Escayg, A., 2001. Identification of epilepsy genes in human and mouse. Annu Rev Genet. 35, 567-588.

Nicolazzo, J. A., Charman, S. A. and Charman, W. N., 2006. Methods to assess drug permeability across the blood-brain barrier. J Pharm Pharmacol. 58, 281-293.

Owen, A., Pirmohamed, M., Tettey, J. N., Morgan, P., Chadwick, D. and Park, B. K., 2001a. Carbamazepine is not a substrate for P-glycoprotein. Br J Clin Pharmacol. 51, 345-349.

Pastan, I., Gottesman, M. M., Ueda, K., Lovelace, E., Rutherford, A. V. and Willingham, M. C., 1988. A retrovirus carrying an MDR1 cDNA confers multidrug resistance and polarized expression of P-glycoprotein in MDCK cells. Proc Natl Acad Sci U S A. 85, 4486-4490.

Paul, S., Belinsky, M. G., Shen, H. and Kruh, G. D., 1996. Structure and in vitro substrate specificity of the murine multidrug resistance-associated protein. Biochemistry. 35, 13647-13655.

Pitkanen, A., Schwartzkroin, P.A., Moshe, S.L., 2006. Models of Seizures and Epilepsy. Elsevier Academic Press.

Potschka, H., Fedrowitz, M. and Loscher, W., 2001. P-glycoprotein and multidrug resistance-associated protein are involved in the regulation of extracellular levels of the major antiepileptic drug carbamazepine in the brain. Neuroreport. 12, 3557-3560.

Potschka, H., Fedrowitz, M. and Loscher, W., 2003. Brain access and anticonvulsant efficacy of carbamazepine, lamotrigine, and felbamate in ABCC2/MRP2-deficient TR- rats. Epilepsia. 44, 1479-1486.

Privitera, M., Ficker, D.M., Welty, E., 2001. Topiramate. In: Wyllie, E. (Ed.), The treatment of epilepsy: Principles and practice. Lippincott Williams and Wilkins.

Racine, R., Newberry, F. and Burnham, W. M., 1975. Post-activation potentiation and the kindling phenomenon. Electroencephalogr Clin Neurophysiol. 39, 261-271.

Redrobe, J. P. and Bourin, M., 1999. Evidence of the activity of lithium on 5-HT1B receptors in the mouse forced swimming test: comparison with carbamazepine and sodium valproate. Psychopharmacology (Berl). 141, 370-377.

Regesta, G. and Tanganelli, P., 1999. Clinical aspects and biological bases of drug-resistant epilepsies. Epilepsy Res. 34, 109-122.

Riban, V., Bouilleret, V., Pham-Le, B. T., Fritschy, J. M., Marescaux, C. and Depaulis, A., 2002. Evolution of hippocampal epileptic activity during the development of

hippocampal sclerosis in a mouse model of temporal lobe epilepsy. Neuroscience. 112, 101-111.

Richardson, J. C. and Simmons, N. L., 1979. Demonstration of protein asymmetries in the plasma membrane of cultured renal (MDCK) epithelial cells by lactoperoxidase-mediated iodination. FEBS Lett. 105, 201-204.

Rivers, F., O'Brien, T. J. and Callaghan, R., 2008. Exploring the possible interaction between anti-epilepsy drugs and multidrug efflux pumps; in vitro observations. Eur J Pharmacol. 598, 1-8.

Rizzi, M., Caccia, S., Guiso, G., Richichi, C., Gorter, J. A., Aronica, E., Aliprandi, M., Bagnati, R., Fanelli, R., D'Incalci, M., Samanin, R. and Vezzani, A., 2002. Limbic seizures induce P-glycoprotein in rodent brain: functional implications for pharmacoresistance. J Neurosci. 22, 5833-5839.

Roberts, L. M., Black, D. S., Raman, C., Woodford, K., Zhou, M., Haggerty, J. E., Yan, A. T., Cwirla, S. E. and Grindstaff, K. K., 2008. Subcellular localization of transporters along the rat blood-brain barrier and blood-cerebral-spinal fluid barrier by in vivo biotinylation. Neuroscience. 155, 423-438.

Rogawski, M. A. and Loscher, W., 2004. The neurobiology of antiepileptic drugs. Nat Rev Neurosci. 5, 553-564.

Rogawski, M. A. and Porter, R. J., 1990. Antiepileptic drugs: pharmacological mechanisms and clinical efficacy with consideration of promising developmental stage compounds. Pharmacol Rev. 42, 223-286.

Rudolph, U., Crestani, F., Benke, D., Brunig, I., Benson, J. A., Fritschy, J. M., Martin, J. R., Bluethmann, H. and Mohler, H., 1999. Benzodiazepine actions mediated by specific gamma-aminobutyric acid(A) receptor subtypes. Nature. 401, 796-800.

Scharfman, H. E., 2007. The neurobiology of epilepsy. Curr Neurol Neurosci Rep. 7, 348-354.

Schinkel, A. H. and Jonker, J. W., 2003. Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview. Adv Drug Deliv Rev. 55, 3-29.

Schinkel, A. H., Smit, J. J., van Tellingen, O., Beijnen, J. H., Wagenaar, E., van Deemter, L., Mol, C. A., van der Valk, M. A., Robanus-Maandag, E. C., te Riele, H. P. and et al., 1994. Disruption of the mouse mdr1a P-glycoprotein gene leads to a deficiency in the blood-brain barrier and to increased sensitivity to drugs. Cell. 77, 491-502.

Schmidt, D. and Loscher, W., 2005. Drug resistance in epilepsy: putative neurobiologic and clinical mechanisms. Epilepsia. 46, 858-877.

Schmutz, M., Brugger, F., Gentsch, C., McLean, M. J. and Olpe, H. R., 1994. Oxcarbazepine: preclinical anticonvulsant profile and putative mechanisms of action. Epilepsia. 35 Suppl 5, S47-50.

Shalinsky, D. R., Jekunen, A. P., Alcaraz, J. E., Christen, R. D., Kim, S., Khatibi, S. and Howell, S. B., 1993. Regulation of initial vinblastine influx by P-glycoprotein. Br J Cancer. 67, 30-36.

Shorvon, S. D., 1996. The epidemiology and treatment of chronic and refractory epilepsy. Epilepsia. 37 Suppl 2, S1-S3.

Sills, G. J., Kwan, P., Butler, E., de Lange, E. C., van den Berg, D. J. and Brodie, M. J., 2002. P-glycoprotein-mediated efflux of antiepileptic drugs: preliminary studies in mdr1a knockout mice. Epilepsy Behav. 3, 427-432.

Sisodiya, S. M., 2003. Mechanisms of antiepileptic drug resistance. Curr Opin Neurol. 16, 197-201.

Sisodiya, S. M., Heffernan, J. and Squier, M. V., 1999. Over-expression of P-glycoprotein in malformations of cortical development. Neuroreport. 10, 3437-3441.

Sisodiya, S. M., Lin, W. R., Harding, B. N., Squier, M. V. and Thom, M., 2002. Drug resistance in epilepsy: expression of drug resistance proteins in common causes of refractory epilepsy. Brain. 125, 22-31.

Sisodiya, S. M., Lin, W. R., Squier, M. V. and Thom, M., 2001. Multidrug-resistance protein 1 in focal cortical dysplasia. Lancet. 357, 42-43.

Soontornmalai, A., 2006. Pharmacoresistance to antiepileptic drugs in mesial temporal lobe epilepsy; distribution and function of P-glycoprotein (MDR) and multidrug resistance associated protein (MRP) family in the blood brain barrier. In: Department of Neurophysiology). University of Birmingham, Birmingham.

Soontornmalai, A., Vlaming, M. L. and Fritschy, J. M., 2006. Differential, strain-specific cellular and subcellular distribution of multidrug transporters in murine choroid plexus and blood-brain barrier. Neuroscience. 138, 159-169.

Stanness, K. A., Neumaier, J. F., Sexton, T. J., Grant, G. A., Emmi, A., Maris, D. O. and Janigro, D., 1999. A new model of the blood--brain barrier: co-culture of neuronal, endothelial and glial cells under dynamic conditions. Neuroreport. 10, 3725-3731.

Stanness, K. A., Westrum, L. E., Fornaciari, E., Mascagni, P., Nelson, J. A., Stenglein, S. G., Myers, T. and Janigro, D., 1997. Morphological and functional characterization of an in vitro blood-brain barrier model. Brain Res. 771, 329-342.

Steinlein, O. K., 2004. Genetic mechanisms that underlie epilepsy. Nat Rev Neurosci. 5, 400-408.

Stephen, L. J., Kwan, P. and Brodie, M. J., 2001. Does the cause of localisation-related epilepsy influence the response to antiepileptic drug treatment? Epilepsia. 42, 357-362.

Strazielle, N. and Ghersi-Egea, J. F., 2000. Choroid plexus in the central nervous system: biology and physiopathology. J Neuropathol Exp Neurol. 59, 561-574.

Thom, M., 2004. Recent advances in the neuropathology of focal lesions in epilepsy. Expert Rev Neurother. 4, 973-984.

Thompson, S. M. and Gahwiler, B. H., 1992. Effects of the GABA uptake inhibitor tiagabine on inhibitory synaptic potentials in rat hippocampal slice cultures. J Neurophysiol. 67, 1698-1701.

Tishler, D. M., Weinberg, K. I., Hinton, D. R., Barbaro, N., Annett, G. M. and Raffel, C., 1995. MDR1 gene expression in brain of patients with medically intractable epilepsy. Epilepsia. 36, 1-6.

Trevor, A. J. and Way, W. L., 1995. Sedative-hypnotic drugs. In: Katzung, B. G. (Ed.), Basic and Clinical Pharmacology. Appleton & Lange, Norwalk, CT, pp. 333-349.

Vaillend, C., Mason, S. E., Cuttle, M. F. and Alger, B. E., 2002. Mechanisms of neuronal hyperexcitability caused by partial inhibition of Na+-K+-ATPases in the rat CA1 hippocampal region. J Neurophysiol. 88, 2963-2978.

van Meer, G. and Simons, K., 1982. Viruses budding from either the apical or the basolateral plasma membrane domain of MDCK cells have unique phospholipid compositions. Embo J. 1, 847-852.

van Vliet, E. A., Redeker, S., Aronica, E., Edelbroek, P. M. and Gorter, J. A., 2005. Expression of multidrug transporters MRP1, MRP2, and BCRP shortly after status epilepticus, during the latent period, and in chronic epileptic rats. Epilepsia. 46, 1569-1580.

Volk, H. A., Burkhardt, K., Potschka, H., Chen, J., Becker, A. and Loscher, W., 2004. Neuronal expression of the drug efflux transporter P-glycoprotein in the rat hippocampus after limbic seizures. Neuroscience. 123, 751-759.

von Bonsdorff, C. H., Fuller, S. D. and Simons, K., 1985. Apical and basolateral endocytosis in Madin-Darby canine kidney (MDCK) cells grown on nitrocellulose filters. Embo J. 4, 2781-2792.

Wang, Q., Rager, J. D., Weinstein, K., Kardos, P. S., Dobson, G. L., Li, J. and Hidalgo, I. J., 2005. Evaluation of the MDR-MDCK cell line as a permeability screen for the blood-brain barrier. Int J Pharm. 288, 349-359.

Wieser, H. G., 2004. ILAE Commission Report. Mesial temporal lobe epilepsy with hippocampal sclerosis. Epilepsia. 45, 695-714.

Zaman, G. J., Flens, M. J., van Leusden, M. R., de Haas, M., Mulder, H. S., Lankelma, J., Pinedo, H. M., Scheper, R. J., Baas, F., Broxterman, H. J. and et al., 1994. The human multidrug resistance-associated protein MRP is a plasma membrane drug-efflux pump. Proc Natl Acad Sci U S A. 91, 8822-8826.

Zhang, L., Ong, W. Y. and Lee, T., 1999. Induction of P-glycoprotein expression in astrocytes following intracerebroventricular kainate injections. Exp Brain Res. 126, 509-516.

Zimmermann, C., van de Wetering, K., van de Steeg, E., Wagenaar, E., Vens, C. and Schinkel, A. H., 2008. Species-dependent transport and modulation properties of human and mouse multidrug resistance protein 2 (MRP2/Mrp2, ABCC2/Abcc2). Drug Metab Dispos. 36, 631-640.

Zimprich, F., Sunder-Plassmann, R., Stogmann, E., Gleiss, A., Dal-Bianco, A., Zimprich, A., Plumer, S., Baumgartner, C. and Mannhalter, C., 2004. Association of an ABCB1 gene haplotype with pharmacoresistance in temporal lobe epilepsy. Neurology. 63, 1087-1089.

Zlokovic, B. V., 2008. The blood-brain barrier in health and chronic neurodegenerative disorders. Neuron. 57, 178-201.

Zolnerciks, J. K., Wooding, C. and Linton, K. J., 2007. Evidence for a Sav1866-like architecture for the human multidrug transporter P-glycoprotein. Faseb J. 21, 3937-3948.

8 Abbreviations

a	apical
ABC	ATP-binding cassette
ABCB	ABC transporter subfamily B
ABCC	ABC transporter subfamily C
ABCG	ABC transporter subfamily G
Ach	achetylcholine, neurotransmitter
ADP	adenosine diphsphate, a nucleotide
AED	antiepileptic drug
ANOVA	analysis of variance
ATP	adenosine triphosphate, a nucleotide
AUC	area under the curve
BBB	blood-brain barrier
BCRP	brest cancer resistant protein
BCSFB	brain-CSF barrier
bl	basolateral
BUI	brain uptake index
CA	cornu ammonis, part of the hippocampus proper
CBZ	carbamazepine, AED
CE	collision energy
CNS	central nervous system
CP	choroid plexus
CSF	cerebrospinal fluid
cTR	corrected transport ratio
CXP	collision cell exit potential
DG	dentate gyrus, part of the hippocampal formation
DMSO	dimethyl sulfoxide
DOX	doxycycline
DP	declustering potential
EEG	electroencephalography
EP	entrance potential
ESI	electrospray ionisation
GABA	γ-amminobutyric acid, neurotransmitter

HPLC	high pressure/performance liquid chromatography
HS	hippocampal sclerosis
ICC	immunocytochemistry
KA	kainic acid or kainate
LC	liquid chromatography
LC-MS/MS	liquid chromatography coupled mass spectrometry
LOD	limit of detection
LOQ	limit of quantification
MDCK cells	Madin-Darby canine kidney cells
MDR	multidrug resistance
MRM	multiple reaction monitoring
MRP	multidrug resistance protein, multidrug resistance-associated protein
MS	mass spectrometry
MSD	membrane spanning domain
NBD	nucleotide binding domain
OAT	organic anion transporter (a family of transporters)
OATP	organic anion transporter protein (a family of transporters)
PBS	phosphate-buffered saline
P-gp	P glycoprotein
RT	room temperature
SE	status epilepticus
TEER	trans-endothelial (or epithelial) electrical resistance
TJ	tight junctions
TLE	temporal lobe epilepsy
TM	transmembrane
TMD	transmembrane domain
TR	transport ratio
WT	wild type

9 Curriculum Vitae

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Baldinger, M.H., Soontornmalai, A., Crestani, F., Fritschy, J.-M., 2009. Role of the multidrug transporter Abcb1a in pharmacoresistance to carbamazepine in a mouse model of temporal lobe epilepsy. Epilepsia. to be submitted.

Abstracts for posters:

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